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The Role of Liquid Chromatography-High Resolution Mass Spectrometry in the Diagnosis and Treatment of Substance Misuse

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THE ROLE OF LIQUID CHROMATOGRAPHY-HIGH RESOLUTION MASS SPECTROMETRY IN THE DIAGNOSIS AND TREATMENT OF SUBSTANCE MISUSE

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Abstract

Substance misuse remains problematic with current concerns being the rise in acute poisoning deaths, particularly opioid-associated, and the ever-widening range of drugs available. Strategies for tackling opioid addiction and opioid related-deaths include researching alternative routes of therapeutic agent administration.

Initial urine screening for substance misuse has traditionally employed immunoassays, with confirmation of specific analytes by chromatographic methods. Liquid chromatography-high resolution mass spectrometry (LC-HRMS) offers untargeted analysis without compromising selectivity, and enables users to ascertain putative elemental compositions of an analyte, retrospectively interrogate data, and to incorporate novel analytes easily. These features enable screening and confirmation of drugs in a single method, and may be advantageous for detecting novel psychoactive substances (NPS).

This thesis aims to investigate the role of LC-HRMS in drug analysis in the clinical setting. A simple system was developed that is capable of detecting a wide range of commonly-encountered drugs and metabolites. Non-selective sample preparation was used to enable detection of as many compounds as possible, but significant matrix effects were observed. Additional information regarding selected NPS was ascertained through retrospective identification of mephedrone metabolites in patient urines, and through later incorporation of ethylphenidate, methylphenidate, and ritalinic acid, into the method.

A separate quantitative LC-HRMS method was developed to facilitate pharmacokinetic studies of naloxone and naltrexone administered through alternative routes. The method was also applied to urine samples, with naloxone-3-glucuronide identified as a potential marker to differentiate between Subutex and Suboxone use.

LC-HRMS has advantages in drug detection, particularly in regard to NPS, and in method development. However, application in the clinical setting is restricted by requirements for high throughput, timely results, and operation to accepted ‘cutoff’ values that introduce awkward compromises in system operation. LC-HRMS may have greater application in the forensic setting where more time is available for the analysis of a single sample.

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Abbreviations

Term	Meaning
2-MMC	2-Methylmethcathinone
3-MMC	3-Methylmethcathinone
6-AM	6-Acetylmorphine (6-monoacetylmorphine)
ACMD	Advisory Council for the Misuse of Drugs
ADHD	Attention Deficit Hyperactivity Disorder
AGC	Automatic Gain Control
AIF	All-ion fragmentation
APCI	Atmospheric Pressure Chemical Ionisation
AUC	Area Under Curve
C18	Octadecyl
CEDIA	Cloned enzyme donor immunoassay
CD	Controlled Drug
CI	Chemical Ionisation
CID	Collison-induced dissociation
C_{max}	Maximum (peak) concentration
CYP	Cytochrome P450
$ddMS^2$	Data-dependent MS^2
DFSA	Drug-Facilitated Sexual Assault
ECD	Electron Capture Detection
ED	Electrochemical Detection
EDDP	2-Ethylidene-1,5-dimethyl-3,3-diphenyl-pyrrolidine
EI	Electron Ionisation
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
EQA	External quality assurance
EQC	External quality control
ESI	Electrospray Ionisation
EWDTS	European workplace drug testing society
FDA/CDER	Food and Drug Administration/Centre for Drug Evaluation and Research
FID	Flame Ionisation Detection
FWHM	Full width half maximum
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
GI	Gastro-Intestinal
GPCR	G protein-coupled receptor
HAT	Heroin-assisted treatment
HCD	Higher-energy collisional dissociation
HILIC	Hydrophillic Interaction Liquid Chromatography
HIV	Human immunodeficiency virus
HPLC	High Performance Liquid Chromatography
HRMS	High Resolution Mass Spectrometry
I.D.	Internal Diameter
IM	Intramuscular
IN	Intranasal
IQC	Internal Quality Control
IS	Internal standard
IV	Intravenous
LC	Liquid Chromatography
LC-MS	Liquid Chromatography-Mass Spectrometry

Term	Meaning
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LLE	Liquid Liquid Extraction
LLoQ	Lower Limit of Quantification
LoD	Limit of Detection
Log P	Partition coefficient
$[M+H]^+$	Molecular Ion
m/z	Mass to charge ratio
MALDI	Matrix-assisted laser desorption ionisation
MDA	Misuse of Drugs Act
MDMA	Methylenedioxymetamfetamine, 'ecstasy'
mmu	milli mass unit
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS^2	Tandem mass spectrometry
NaCl	Sodium Chloride
NAS	Neonatal abstinence syndrome
NIDA	National Institute on Drug Abuse
NPS	Novel Psychoactive Substances
OST	Opioid substitution therapy
OT	Orbitrap
OTC	Over-the-counter
PFP	Pentafluorophenyl
PK	Pharmacokinetic
*p*Ka	Acid dissociation constant
ppm	Parts per million
Q-TOF	Quadrupole time-of-flight
QQQ	Triple quadrupole
rpm	Rotations per minute
RSD	Relative standard deviation
RT	Retention Time
SC	Subcutaneous
SIM	Selected Ion Monitoring
SPE	Solid Phase Extraction
SRM	Selected Reaction Monitoring
STA	Systematic toxicological analysis
$t_{1/2}$	Half-life
TCA	Trichloroacetic Acid
TDM	Therapeutic Drug Monitoring
THC	Tetrahydrocannabinol
TIC	Total Ion Chromatogram
TLC	Thin Layer Chromatography
T_{max}	Time at which C_{max} is observed
TOF	Time-of-flight
UGT	Uridine 5'-diphospho-glucuronosyltransferase
UV	Ultra-violet
v/v	Volume/volume
w/v	Weight/volume
WHO	World Health Organisation
XIC	Extracted Ion Chromatogram

1 Introduction

1.1 Substance Misuse

Substance misuse refers to the harmful use of generally psychoactive substances, including alcohol, tobacco, and illicit drugs. The International Classification of Diseases (ICD-10) defines 'harmful use' when there is evidence that substance use is causing physical or psychological harm. It defines 'drug dependence' as occurring if three or more indicators of dependence (Table 1.1) have been present for at least one month within the past year (World Health Organization, 1993).

Table 1.1 – Indicators of drug dependence according to the International Classification of Diseases (World Health Organization, 1993)

Dependence Syndrome Manifestations
Strong desire or compulsion to take the substance
Impaired capacity to control substance-taking behaviour in terms of termination or level of use
A physiological withdrawal state when substance-use is reduced or ceased
Evidence of tolerance to the effects of the substance
Preoccupation with substance use
Persistent substance use despite clear evidence of harmful consequences

The economic cost of drug dependence has been estimated as £15 billion per year in Britain (The Centre for Social Justice, 2013), and there are obviously important health and social issues too. There is an urgent need for improvement in the management of substance misuse-related disorders, and thus it is important to invest in research for improvement of substance misuse prevention and treatment strategies. Currently two of the main concerns relating to drug misuse are the rise in acute poisoning deaths (particularly associated with opioids), and the dynamic and constantly changing market for novel psychoactive substances (NPS, EMCDDA, 2016). Tackling these issues is multi-disciplinary, ranging from implementation of political policies and optimisation of treatment to detection and monitoring of drug misuse.

1.1.1 Legal Classification of Drugs

The legal classification of drugs in the United Kingdom (UK) is largely governed by the Misuse of Drugs Act (1971) (MDA). The act was introduced with the main purpose of preventing the non-medical use of certain drugs, with drugs subject to this act termed 'controlled drugs' (CD). The Act denotes three classes for CD, broadly based on the harmfulness attributable to a substance, with Class A drugs having the greatest penalties associated when supplying or possessing these compounds (Table 1.2a). The regulations

for supply and possession of CD are given in the Misuse of Drugs Regulations (2001), which delineates five schedules (Table 1.2b). There has been debate over whether the MDA classification accurately reflects the harm associated with a substance, and additionally that the legal status of a substance does not correlate with its perceived harm. Most notably, alcohol and tobacco (both legal substances with controlled sales) have greater perceived harm than some Class A drugs (Nutt *et al.*, 2010; Taylor *et al.*, 2012).

Table 1.2 – Controlled drug legislation according to a) the Misuse of Drugs Act 1971 c. 38, and b) the Misuse of Drugs Regulations 2001 No. 3998

a)		
Drug Class	**Legal Penalties for i) possession, and ii) supply/possession with intent to supply**	**Examples**
A	i) 6 months – 7 years, £5,000 – unlimited fine ii) 6 months – life imprisonment, £5,000 – unlimited fine	Cocaine Diamorphine/Morphine Methadone Metamfetamine
B	i) 3 months – 5 years, £2,500 – unlimited fine ii) 6 months – 14 years, £5,000 – unlimited fine	Amfetamine Barbiturates Cannabis Codeine Ketamine
C	i) 3 months – 2 years, £500 – unlimited fine ii) 3 months – 14 years, £2,000 – unlimited fine	Buprenorphine Most benzodiazepines Tramadol Z-drugs Anabolic steroids
b)		
Drug Schedule	**Medicinal requirements**	**Examples**
1	No medical use Possession & supply prohibited	Lysergic acid diethylamide (LSD)
2	Full CD requirements Safe custody requirement Requirement to maintain registers	Diamorphine Amfetamines Cocaine
3	Same prescription requirements as Schedule 2 No requirement to maintain registers Some drugs require safe custody	Most barbiturates Buprenorphine Tramadol
4 (Part I)	No CD prescription requirements No safe custody requirements	Most benzodiazepines Z-drugs
4 (Part II)		Anabolic steroids
5	Exempt from all requirements due to low strength Need to retain invoices for 2 years	Codeine (<100mg per dosage unit)

Over the last decade, NPS have emerged. Most of these drugs are produced through altering the chemical structure of well-known psychoactive substances, e.g. methylenedioxymetamfetamine (MDMA, 'ecstasy'). Through these structural modifications, the novel compounds often by-passed existing controlled drug legislation. In an attempt to control these substances, the British Government introduced a 'temporary class drug order' which enabled substances to swiftly be brought under the MDA and placed under temporary control for up to 12 months. During this time the Advisory Council for the Misuse of Drugs (ACMD) decide whether permanent control is required or not. It does not make possession for personal use illegal, but does give police the authority to confiscate and destroy these substances. It also makes import, distribution and sale of the substance illegal and these actions can lead to a fine or a prison sentence (Home Office, 2011). Further to this, the Psychoactive Substances Act (2016) was introduced to restrict the production, sale and supply of all psychoactive substances. The only psychoactive substances exempt from this act are substances ordinarily consumed as food (e.g. nutmeg), nicotine and tobacco products, caffeine, alcohol, medicinal products as defined by The Human Medicines Regulations (2012), and drugs that are already controlled by the MDA (1971). The act also affects responsible retailers who supply products that contain psychoactive substances, e.g. solvents and butane. In doing so, it has replaced the Intoxicating Substances (Supply) Act (1985).

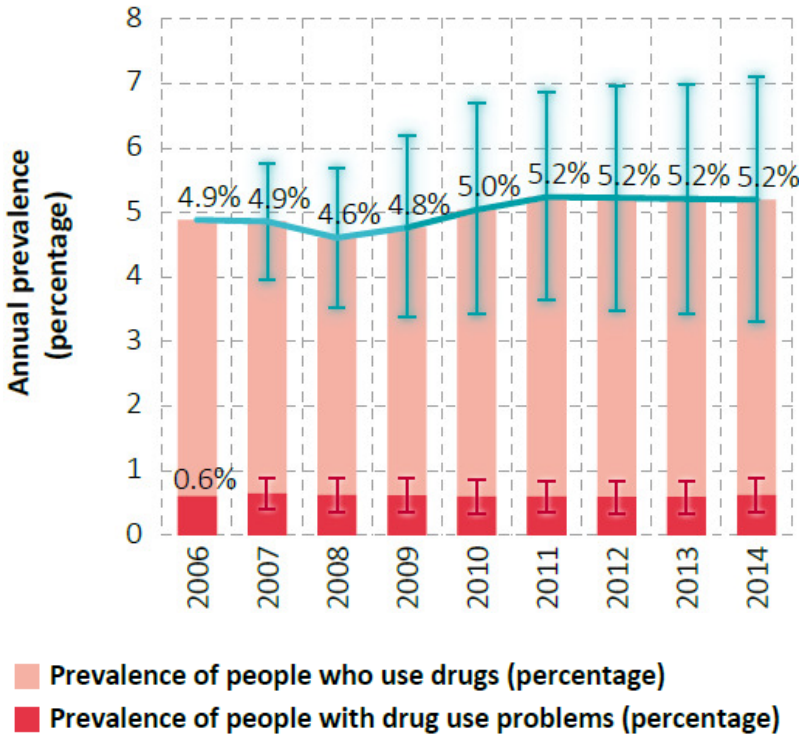
The legal classification of drugs varies between countries, with the main difference in legal status noted for cannabis. Cannabis has been legalised for recreational use in 8 states in the USA, and other countries (e.g. the Netherlands) have more lenient laws for personal use.

1.1.2 Prevalence of Substance Misuse

Globally, the prevalence of drug misuse has remained fairly stable over the last few years (Figure 1.1). Cannabis is consistently the most widely used illicit drug, with an estimated 183 million people having used the drug in 2014, equating to 3.8 % of the world's adult population (United Nations Office on Drugs and Crime, 2016). The global prevalence of the misuse of opioids such as heroin (impure diamorphine) is estimated at 0.7 %, ranking opioids as the third most prevalent drug class misused, behind cannabis and amfetamines (United Nations Office on Drugs and Crime, 2016). The global estimate of opioid dependent individuals (aged 15 years or older) was 10.4 million in 1990, rising to 15.5 million in 2010 (Degenhardt *et al.*, 2014).

<u>Figure 1.1</u> – Global trends in the estimated prevalence of drug misuse (15-64 year-olds), 2006-2014

Taken from the World drug report (United Nations Office on Drugs and Crime, 2016).



Opioid addiction remains a problem within the UK, and many users are reliant on prescribed opioid substitutes, with 17.3 million and 2.6 million prescriptions for methadone and buprenorphine, respectively, issued in England and Wales between 2007-2012 (Marteau *et al.*, 2015). Whilst the prevalence of opioid misuse is relatively low, opioids feature in the greatest number of drug-related deaths (Table 1.3). In addition, UK deaths involving heroin or morphine have increased by 64 % from 2012 to 2014 (Wise, 2015). This increase has also been observed in the USA, with heroin-related deaths rising by 39 % from 2013 to 2014 (United Nations Office on Drugs and Crimes, 2015). Accurate identification of deaths due to heroin administration may be hindered by the rapid metabolism of diamorphine (the principal active component of heroin) and its unique metabolite 6-acetylmorphine (6-AM), meaning morphine may be the only compound detected in samples of body fluids or tissues obtained post-mortem (Figure 1.2). In this situation administration of heroin as opposed to morphine cannot be distinguished from analytical results alone, unless other markers of heroin use such as meconin or 6-acetylcodeine are also present.

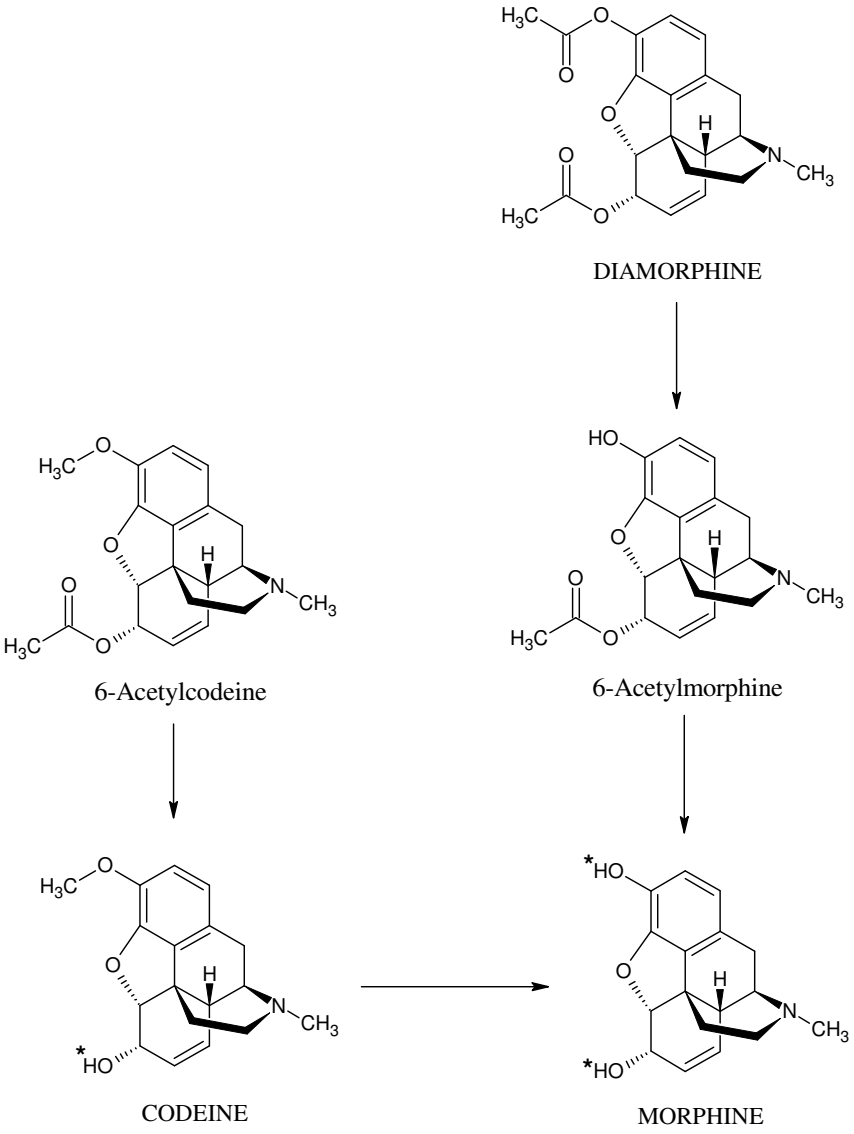
Table 1.3 – Drug use prevalence in England and Wales (Home Office, 2015) and drug-related deaths (Office for National Statistics, 2016)

Drug-related deaths are counted when the named drug or drug group was detected; other drugs and/or alcohol may also have been detected in some cases.

Drug	Prevalence of use in general population (use in the last year, age 16-59 y)	Number of deaths in 2015
Cannabis	6.7 %	21
Cocaine	2.4 %	320
MDMA	1.7 %	57
Amfetamine	0.6 %	90
Opioids (Diamorphine/morphine)	0.1 %	1,989 (1,201)

Figure 1.2 – Metabolic pathway of diamorphine, morphine and codeine

Parent drugs are shown capitalised; * site of glucuronidation.



1.1.3 Opioid Receptors and Pharmacology

Opioid receptors are a group of inhibitory G protein-coupled receptors (GPCR) distributed widely through the brain, spinal cord and digestive tract. They form part of an endogenous system with three main classes of opioids being present in the body: endorphins, enkephalins, and dynorphins. These opioids act as neurotransmitters and neuromodulators at the three major subclasses of 'classical' opioid receptor: delta (δ), kappa (κ), and mu (μ), to produce analgesia. Opioid drugs act at these same receptors, and are classified according to their receptor interaction into four groups: agonists, antagonists, partial agonists and agonist-antagonists (Table 1.4). Two other opioid receptors exist; the nociceptin receptor and the opioid growth factor receptor. However, these share little sequence similarity to the 'classical' receptors and possess little or no affinity for opioids (Butour *et al.*, 1997).

Table 1.4 – Classification of opioid drugs

Agent Class	Example/s	Action
Agonist	Morphine Methadone	Activation of all receptor subclasses, though with different affinity
Antagonist	Naloxone Naltrexone	Devoid of agonist activity at all receptor subclasses; may displace an agonist from a receptor
Partial agonist	Buprenorphine	Agonist activity at one or more, but not all receptor subclasses
Agonist-antagonist	Nalorphine	Agonist activity at one type and antagonist activity at another type of receptor subclass

When taken orally, most opioids have low bioavailability meaning that only a small proportion of the dose reaches the brain and elicits the desired effect. This is a result of first-pass metabolism where much of the drug is metabolised in the intestine and/or the liver prior to reaching the systemic circulation. Drug bioavailability may be increased by administration directly into the systemic circulation. Those who abuse opioid agonists typically inject or inhale the drug in order to 'achieve a greater high'. Opioid antagonists may also be given by non-oral routes to improve bioavailability, e.g. naloxone is typically injected.

Opioid tolerance, dependence, and addiction are all manifestations of changes to the brain as a result of chronic opioid use. Opioid agonists activate the mesolimbic pathway (the reward pathway) causing dopamine release in part of the brain (the nucleus

accumbens), eliciting a feeling of pleasure (Kosten and George, 2002). The constant overstimulation of this pathway plays a central role in the neurobiology of opioid addiction. Repeated opioid exposure alters the brain so that it may function apparently normally when opioid agonists are present, and functions abnormally when they are absent. Clinically these alterations cause opioid tolerance, where a higher dose is required to achieve the same opioid effect, and opioid dependence, where an individual will experience withdrawal on cessation of opioid administration. Withdrawal has two stages, the acute stage where mainly physical features predominate (e.g. nausea, vomiting, abdominal cramps, diarrhoea), and the post-acute stage where symptoms are mainly psychological (e.g. anxiety, depression, irritability) and have a longer duration.

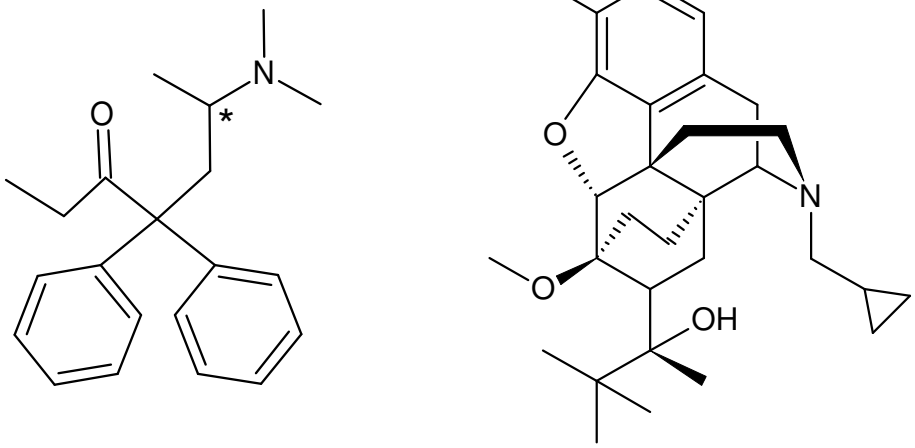
Opioid misuse falls into two categories; illicit drug misuse (e.g. heroin), and prescription drug misuse (e.g. oxycodone) where opioids may be obtained through legal or illegal routes. These two categories often cross-over, for example heroin users may use prescription opioids to prevent withdrawal if heroin is not available. Awareness of prescription medicine misuse has increased over the last few years. It has been most pronounced in the USA, where opioid prescriptions increased from 76 million in 1991 to 207 million in 2013 (National Institute on Drug Abuse, 2014), and prescription drug misuse is second only to cannabis use across all age groups (Hernandez and Nelson, 2010; Hughes *et al.*, 2016). There is also evidence of problematic opioid analgesic use outside the USA, particularly in Europe and Australia (Morley *et al.*, 2017). Whilst misuse of prescription medication is not currently thought to be as serious an issue in the UK, data suggest that there may be a trend towards the situation in the USA that warrants attention (Giraudon *et al.*, 2013; Morley *et al.*, 2017; Weisberg *et al.*, 2014).

Strategies for tackling opioid addiction and opioid related-deaths can be at two levels: to reduce associated deaths through greater provision of the antidote (naloxone), and to research further into improvement of maintenance/detoxification treatments. The most effective pharmacological approach to the treatment of opioid dependency is opioid substitution therapy (OST) using agonists. OST has been shown to decrease the adverse consequences of heroin use, including drug-related crime and HIV risk (Cone and Preston, 2002). Buprenorphine and methadone (Figure 1.3) are both used for OST, and are effective for the treatment of heroin dependence (Mattick *et al.*, 2009, 2014). Methadone is a chiral compound and is prescribed as a racemic mixture, however only *R*-methadone is responsible for therapeutic effect through its affinity for μ opioid receptors. The *S*-enantiomer is a poor μ agonist, and has been postulated to be

responsible for adverse cardiac effects associated with methadone use (Lin *et al.*, 2009). Buprenorphine also acts at the μ opioid receptor, but possesses additional antagonist activity at κ opioid receptors. Through their agonist activity both methadone and buprenorphine can act as substitutes for heroin. OST is available in most European countries (Table 1.5). Methadone use is more prevalent in the UK; however other countries favour the use of buprenorphine (e.g. France, Turkey). In 2005, the World Health Organisation (WHO) added methadone and buprenorphine to the WHO Model List of Essential Medicines for opioid addiction treatment (World Health Organisation, 2005).

Figure 1.3 – Structures of opioid agonists used for treatment of opioid dependency

* chiral centre



Methadone Buprenorphine

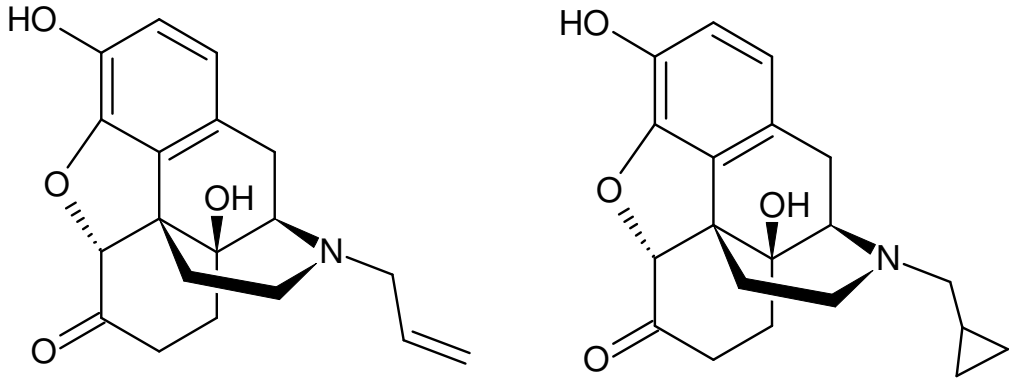
Unfortunately, side effects such as constipation, excess sweating, drowsiness and decreased libido are associated with OST (Tetrault and Fiellin, 2012). In addition, there is a risk of acute poisoning, particularly with methadone. Another disadvantage of agonist therapy is that prescribed agonist drugs may be sold illicitly so that the user may purchase their preferred drug. Diversion of opioid agonists used for maintenance treatment is a challenge faced worldwide (Dasgupta *et al.*, 2010; Johnson and Richert, 2015a; Larance *et al.*, 2011; Nordmann *et al.*, 2012). Studies report between a fifth and a quarter of individuals prescribed maintenance therapy have diverted their medication (Johnson and Richert, 2015b; Larance *et al.*, 2011). In some countries opioid agonists developed for treatment purposes have now become the most widely-abused opioid in that country, e.g. buprenorphine in Finland (Aalto *et al.*, 2007; EMCDDA, 2005; Launonen *et al.*, 2015).

Table 1.5 – Summary of the availability of opioid substitution therapy across Europe (Data from EMCDDA, 2013)

Country	Year opioid substitution therapy was introduced	Most common form of substitution therapy
Austria	1987	Slow-release oral morphine
Belgium	2002	Methadone
Bulgaria	1995	Methadone
Croatia	1991	Buprenorphine
Cyprus	2007	Buprenorphine
Czech Republic	1998	Buprenorphine
Denmark	1970	Methadone
Estonia	2001	Methadone
Finland	1974	Buprenorphine
France	1995	Buprenorphine
Germany	1992	Methadone
Greece	1993	Buprenorphine
Hungary	1994	Methadone
Italy	1975	Methadone
Ireland	1992	Methadone
Latvia	1996	Methadone
Lithuania	1995	Methadone
Luxembourg	2002	Methadone
Malta	1987	Methadone
Netherlands	1968	Methadone
Norway	1998	Buprenorphine
Poland	1993	Methadone
Portugal	1977	Methadone
Romania	1998	Methadone
Slovakia	1997	Methadone
Slovenia	1990	Methadone
Spain	1990	Methadone
Sweden	1967	Information not available
Turkey	2010	Buprenorphine
United Kingdom	1968	Methadone

Naloxone and naltrexone (Figure 1.4) both act as antagonists at opioid receptors. Both may interact with all receptor subclasses (Shader, 2003), but have greatest affinity at μ opioid receptors (Helm *et al.*, 2008). At present the main clinical use of antagonists, principally naloxone, is in treatment of acute opioid overdose. Antagonist-based treatment for opioid dependency is not widely used, with the major criticism of this strategy being short retention times in treatment, and increased risk of overdose due to reduced tolerance to opioids in the event of relapse.

<u>Figure 1.4</u> – Structure of opioid antagonists used for treatment of opioid overdose and dependency



Naloxone Naltrexone

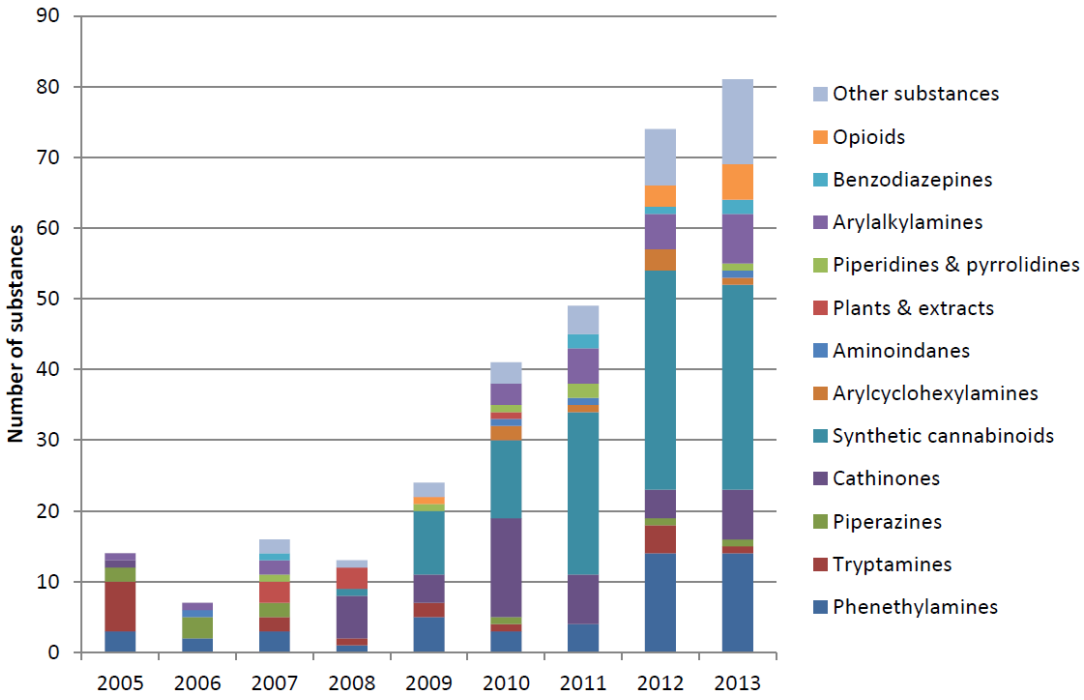
When compared to agonist therapy, antagonists offer some significant benefits including no abuse potential, and no tolerance associated with use. In addition, antagonists are associated with minimal adverse effects. Socially antagonist therapy may be deemed a more acceptable approach to treatment of opioid dependency, and in some countries is the only treatment option (e.g. Russia, Krupitsky *et al.*, 2010).

Antagonists, namely naloxone, have also been utilised to deter opioid abuse through co-formulation with partial agonists or agonists such as buprenorphine and oxycodone (Colucci *et al.*, 2014; DePriest and Miller, 2014; Gershell and Goater, 2006; Schaeffer, 2012; Soyka, 2015; Vocci *et al.*, 2005). When these preparations are taken as intended (orally or sublingually) they have the desired effect due to the low oral bioavailability of naloxone, however when misused (e.g. injected or insufflated, i.e. 'snorted') the naloxone reaches sufficient concentration at the receptors to block the effect of the opioid and induce symptoms of opioid withdrawal. Co-formulation has also been explored to try and reduce undesirable side effects of agonists, such as constipation, associated with therapeutic opioid administration through addition of a neutral antagonist (e.g. 6-β-naltrexol) that preferentially antagonises opioid effects on the gastrointestinal transit compared to effects on the central nervous system (Mendelson *et al.*, 2011).

1.1.4 The Changing Drug Scene

Over the last decade there has been a rapid and continuous growth in the use of NPS ('legal highs', 'designer drugs', 'research chemicals') (Figure 1.5). Typically these products have been marketed as 'plant food' or 'herbal incense' not intended for human consumption. These products mimic the psychoactive effects of drugs of abuse, but were not illegal due to slight structural deviations from controlled drugs as discussed previously. Due to the uncontrolled nature of production, NPS marketed as containing a particular substance often do not contain the compound specified, but rather a variety of other substances, and in some cases may only contain caffeine (Brandt *et al.*, 2010). As a result, individuals are unaware of what they actually are taking and the potency of the compound(s) ingested. In addition, as little or no pharmacological or toxicological information exists for these substances long-term risks, in addition to short-term risks, remain unknown. The majority of emerging NPS are either synthetic cannabinoids, or cathinone derivatives, although derivatives of other drugs such as benzodiazepines and opioids are also marketed.

Figure 1.5 – Number and main groups of NPS notified for the first time to the EMCDDA Early Warning System, 2005-2013 (Taken from Stephenson and Richardson, 2014).



1.1.4.1 Novel Stimulants

Mephedrone (4-methylmethcathinone, '4-MMC', 'Meow Meow', 'Miaow', 'White Magic', 'M-Cat', 'Bubble') was one of the first NPS to appear and has been one of the most publicised, being reported by the UK media in March 2010 following several deaths said to be associated with the drug. Mephedrone was portrayed as a 'legal' alternative to MDMA. On 16 April 2010, mephedrone and other substituted cathinones became classified as Class B drugs (The Misuse of Drugs Act 1971 (Amendment) Order, 2010). However, many novel stimulants have been developed since that time and continue to be sold (Table 1.6).

As well as structural alteration of illicit psychoactive drugs, alteration of pharmaceutical drugs with stimulant properties has been reported. Methylphenidate is a psychostimulant prescribed as first-line treatment in Attention Deficit Hyperactivity Disorder (ADHD), but may also be abused for its stimulant properties. When methylphenidate is taken concurrently with ethanol, *S,S*-ethylphenidate may be formed by enantioselective transesterification (Dinis-Oliveira, 2017; Patrick *et al.*, 2013). More recently, ethylphenidate has been marketed as a 'legal high' (Ho *et al.*, 2015; Krueger *et al.*, 2014). Details of six other phenidate analogues marketed as 'legal highs' have been published (Klare *et al.*, 2017; Markowitz *et al.*, 2013).

1.1.4.2 Synthetic Cannabinoids

Synthetic cannabinoids ('Spice', 'K2') are highly potent drugs that bind to the same receptors as Δ_9-tetrahydrocannabinol (Δ_9-THC), the primary psychoactive substance in cannabis. Many of the synthetic cannabinoids were originally synthesised as pharmacological probes for investigating the endogenous cannabinoid system and developing potential pharmacotherapies (Diao and Huestis, 2017). A huge range of synthetic cannabinoids exist with minor structural changes between compounds (Table 1.7). By April 2015, 858 synthetic cannabinoids had been scheduled in Japan (Uchiyama *et al.*, 2015), and many more are likely to be developed. Considerable morbidity and mortality has been associated with the use of synthetic cannabinoids, with the risk of hospital admission estimated as 30 times higher than that associated with cannabis (Winstock *et al.*, 2015).

Table 1.6 – Chemical structures and names of selected novel stimulants

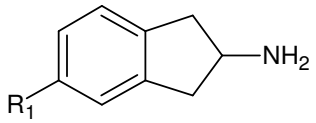
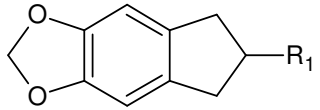
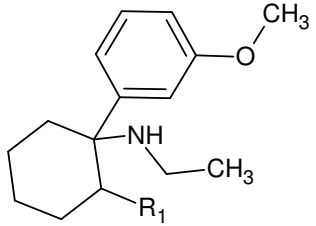
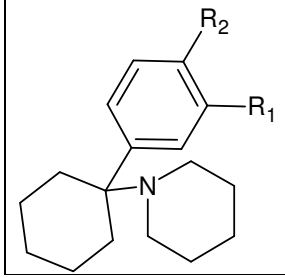
Class and Chemical Structure		Name
Aminoindanes		
	$R_1 = H$ $R_1 = I$	2-Aminoindane (2-AI) 5-Iodo-2-aminoindane (5-IAI)
	$R_1 = NH_2$ $R_1 = NH\text{-}CH_3$	5,6-Methylenedioxy-2-aminoindane (MDAI) 5,6-Methylenedioxy-*N*-methyl-2-aminoindane (MDMAI)
Arylcyclohexylamines		
	$R_1 = O$ $R_1 = H$	Methoxetamine 3-Methoxyeticyclidine (3-MeO-PCE)
	$R_1 = O\text{-}CH_3$, $R_2 = H$ $R_1 = H$, $R_2 = O\text{-}CH_3$	3-Methoxyphencyclidine (3-MeO-PCP) 4-Methoxyphencyclidine (4-MeO-PCP)

Table 1.6 (cont.) – Chemical structures and names of selected novel stimulants

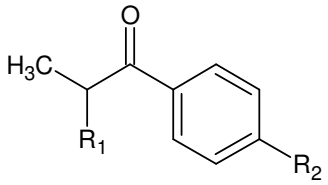
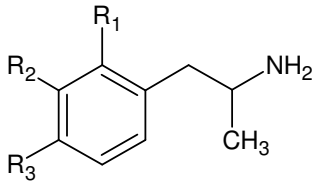
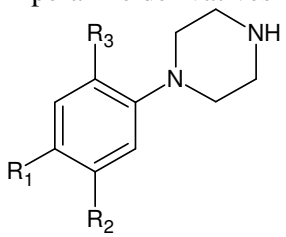
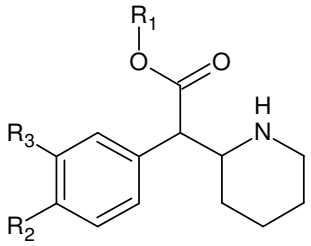
Class and Chemical Structure		Name
Cathinones 	$R_1 = NH_2$, $R_2 = H$ $R_1 = NH\text{-}CH_3$, $R_2 = H$ $R_1 = NH\text{-}CH_3$, $R_2 = CH_3$	Cathinone ('khat') Methcathinone 4-Methylmethcathinone (mephedrone, 4-MMC)
Phenethylamines 	$R_1 = F$, $R_2 = H$, $R_3 = H$ $R_1 = H$, $R_2 = F$, $R_3 = H$ $R_1 = H$, $R_2 = H$, $R_3 = F$ $R_1 = O\text{-}CH_3$, $R_2 = H$, $R_3 = H$ $R_1 = H$, $R_2 = O\text{-}CH_3$, $R_3 = H$ $R_1 = H$, $R_2 = H$, $R_3 = O\text{-}CH_3$	2-Fluoroamfetamine (2-FA) 3-Fluoroamfetamine (3-FA) 4-Fluoroamfetamine (4-FA) 2-Methoxyamfetamine (2-MA) 3-Methoxyamfetamine (3-MA) 4-Methoxyamfetamine (paramethoxyamfetamine, PMA, 4-MA)
Piperazine derivatives 	$R_1 = H$, $R_2 = H$, $R_3 = H$ $R_1 = O\text{-}CH_3$, $R_2 = H$, $R_3 = H$ $R_1 = Cl$, $R_2 = H$, $R_3 = H$ $R_1 = H$, $R_2 = Cl$, $R_3 = H$ $R_1 = O\text{-}CH_2\text{-}O$, $R_2 = O\text{-}CH_2\text{-}O$, $R_3 = H$	Benzylpiperazine (BZP) 4-Methoxyphenylpiperazine (MeOPP) 4-Chlorophenylpiperazine (pCPP) 3-Chlorophenylpiperazine (mCPP) 3,4-Methylenedioxybenzylpiperazine (MDBP)
Phenidate derivatives 	$R_1 = C_2H_5$, $R_2 = H$, $R_3 = H$ $R_1 = CH_3$, $R_2 = CH_3$, $R_3 = H$ $R_1 = CH_3$, $R_2 = Cl$, $R_3 = Cl$ $R_1 = C_2H_5$, $R_2 = Cl$, $R_3 = Cl$ $R_1 = CH(CH_3)_2$, $R_2 = H$, $R_3 = H$	Ethylphenidate 4-Methylmethylphenidate 3,4-Dichloromethylphenidate 3,4-Dichloroethylphenidate Isopropylphenidate

Table 1.7 – Chemical structures and names of selected synthetic cannabinoids

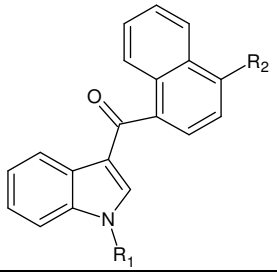
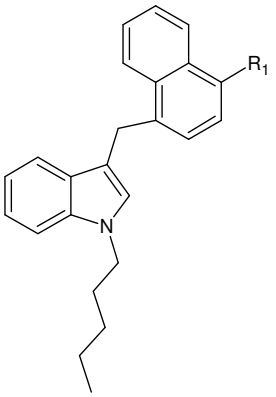
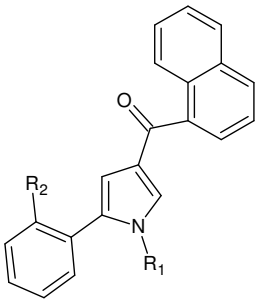
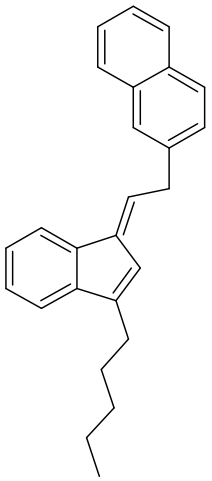
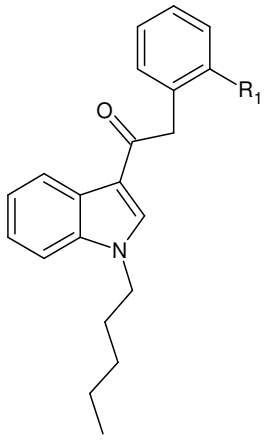
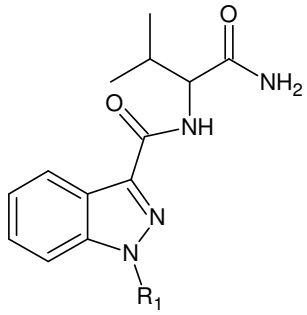
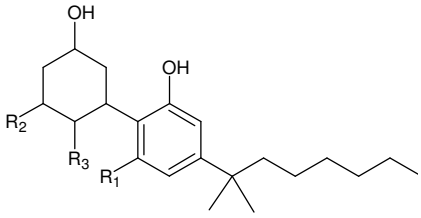
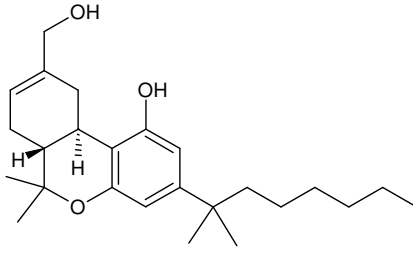
Class and Chemical Structure		Name
Naphthoylindoles 	$R_1 = C_5H_{11}$, $R_2 = H$ $R_1 = CH_3$, $R_2 = H$ $R_1 = C_4H_9$, $R_2 = H$ $R_1 = C_5H_{11}$, $R_2 = Cl$ $R_1 = C_5H_{10}F$, $R_2 = H$	JWH-018 JWH-070 JWH-073 JWH-398 AM-2201
Naphthylmethylindoles 	$R_1 = H$ $R_1 = CH_3$ $R_1 = O\text{-}CH_3$	JWH-175 JWH-184 JWH-185
Naphthoylpyrroles 	$R_1 = H$, $R_2 = C_6H_{13}$ $R_1 = F$, $R_2 = C_5H_{11}$	JWH-147 JWH-307
Naphthylmethylindenes 		JWH-176

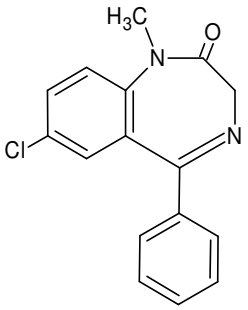
Table 1.7 (cont.) – Chemical structures and names of selected synthetic cannabinoids

Class and Chemical Structure		Name
Phenylacetylindoles 	R_1 = H R_1 = O-CH_3 R_1 = CH_3 R_1 = F R_1 = Cl R_1 = Br	JWH-167 JWH-250 JWH-251 JWH-311 JWH-203 JWH-249
Indazoles 	R_1 = CH_2-Ph R_1 = $C_5H_{10}F$ R_1 = CH_2-PhF	AB-CHMINACA 5F-AB-PINACA AB-FUBINACA
Cyclohexylphenols 	R_1 = H, R_2 = H, R_3 = H R_1 = OH, R_2 = $(CH_3)_2$, R_3 = H R_1 = H, R_2 = H, R_3 = C_3H_6OH	CP 47,497 O-1871 CP 55,940
Classical cannabinoids 		HU-210

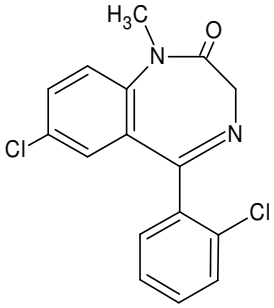
1.1.4.3 Designer Benzodiazepines

Benzodiazepines are widely misused for their sedative and hypnotic effects, typically as part of a poly-drug abuse pattern. Cocaine users often take benzodiazepines to ameliorate the 'crash' during withdrawal, which may give rise to symptoms such as anxiety, dysphoria and insomnia (Sofuoglu *et al.*, 2005). Opiate users commonly take benzodiazepines to enhance the 'high', with use of diazepam by heroin addicts prevalent nowadays (Fatséas *et al.*, 2009; Vogel *et al.*, 2013). Benzodiazepines licensed for use in countries outside of the UK have been sold as 'legal highs' within the UK, in particular phenazepam (Maskell *et al.*, 2011b). More recently, designer benzodiazepines have become a rapidly growing class of drugs of abuse in their own right. The first designer benzodiazepines to become available online were diclazepam, flubromazepam, and pyrazolam (Figure 1.6). Most of these compounds were originally synthesised as drug candidates by pharmaceutical companies, but none are approved for medicinal use in any country (Moosmann *et al.*, 2015). Active metabolites of benzodiazepines have been marketed as designer drugs, e.g. fonazepam (desmethylflunitrazepam) and nifoxipam (3-hydroxydesmethylflunitrazepam) are both active metabolites of flunitrazepam (Katselou *et al.*, 2017).

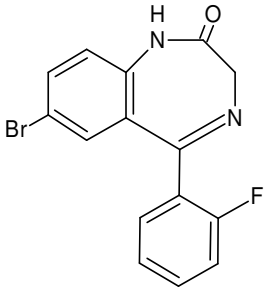
Figure 1.6 – Comparison of the chemical structures of selected designer benzodiazepine drugs to licenced benzodiazepines (diazepam and alprazolam)



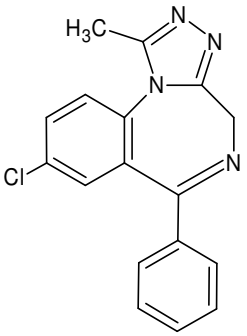
Diazepam



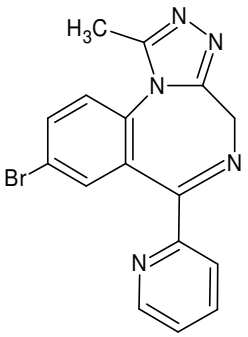
Diclazepam



Flubromazepam



Alprazolam



Pyrazolam

1.1.4.4 Designer Opioids

The number of new opioids recorded since 2009 is much lower (less than 20) in comparison to other classes of NPS. Synthetic opioids include MT-45, U-47700, AH-7921 and fentanyl derivatives. Both MT-45 and AH-7921 ('Doxylam') were originally synthesised in the 1970s by pharmaceutical companies. Both compounds are thought to have similar potency to morphine (Zawilska and Andrzejczak, 2015). Numerous fentanyl derivatives have been reported over the last few years (Helander *et al.*, 2016, 2017; Zawilska, 2017). The fentanyl derivatives pose an especially serious concern for public health because of their high potency (Table 1.8) and because they are often sold under the guise of, or mixed with, heroin to unsuspecting users. Clusters of opioid overdose and deaths due to fentanyl-adulterated heroin have been reported in Australia (Rodda *et al.*, 2017), and in the USA (Bode *et al.*, 2017). Acetylfentanyl has also been sold as oxycodone pills (Stogner, 2014), and fentanyl and U-47700 have been sold as 'Norco' (hydrocodone and paracetamol) tablets (Armenian *et al.*, 2017; Sutter *et al.*, 2017). In these cases, overdose is likely and if not treated may cause death. Naloxone will reverse overdoses; however higher dosing and prolonged infusion may be required (Sutter *et al.*, 2017). Most fentanyl analogues exhibit extensive metabolism, meaning that identification and detection of metabolites as opposed to the parent drug is important, particularly if there is a delay between consumption and sampling (Allibe *et al.*, 2017).

Table 1.8 – Comparison of the relative potency of fentanyl and selected derivatives to morphine

Data from Higashikawa and Suzuki, 2008
ED_{50} – median effective dose, LD_{50} – median lethal dose

Compound	ED_{50} (mg/kg)	LD_{50} (mg/kg)	Potency ratio to morphine
Fentanyl	0.0061	62	54
Acetylfentanyl	0.021	9.3	16
Butyrylfentanyl	0.047	-	7
3-Methylfentanyl	0.00058-0.0068	-	49-569

1.2 The Role of the Clinical Toxicology Laboratory in Drug Testing

1.2.1 Poisoning

Acute poisoning is a relatively common reason for presentation to hospital. Toxicological analyses can provide important information for certain drugs (e.g. paracetamol, lithium) which guides the clinical management of the patient. In other cases, patient treatment is guided by the toxidrome, i.e. the features the patient presents with, and specific identification of the toxin may not be immediately necessary. This is particularly true for many illicit drugs where no specific antidote exists, and treatment typically consists of supportive measures. Opioid toxicity has a very specific toxidrome of coma, pinpoint pupils and respiratory depression, and can be reversed through administration of naloxone in adequate dosage. Later identification of the toxin by the laboratory may help to confirm the diagnosis and guide longer term treatment of the patient.

On the other hand, toxicological analyses can play a useful role in the diagnosis of poisoning when it may not be suspected, where the use of certain antidotes is being contemplated, or where the use of active elimination therapy is being considered (Flanagan and Watson, 2009). It may also be relevant in identifying cases of Munchausen Syndrome or Munchausen-by-proxy, where a poisoning may be a result of self-medication or inappropriate drug administration by a carer, respectively (Holstege and Dobmeier, 2006).

1.2.2 Identification of Unknown Substances

Analysis of unknown substances may be useful in different scenarios. Identifying substances (e.g. tablets, powders) found on a patient through toxicological analyses may aid clinical management in cases of poisoning, particularly if the patient is unconscious. Drug identification may be particularly relevant when it is a NPS, as even the user may be unaware of what drug they have taken so direct analysis of the substance can provide conclusive results. Substance identification may also be of use when an individual is taking non-prescribed medications (e.g. dietary supplements, herbal remedies) as the analysis may aid the clinician in discerning the cause of a change in health.

1.2.3 Treatment of Drug Addiction

Use of toxicological analyses can aid treatment of drug addiction through providing the clinician with accurate information as to the current drug use of an individual. The results can be used to identify if drugs are still being misused, and also to assess whether a patient is adhering to prescribed medication. In terms of drug dependency, this may involve substitution or detoxification medication (e.g. methadone or buprenorphine for opioid dependency) or medication to ease withdrawal symptoms (e.g. antispasmodics such as mebeverine, and anxiolytics such as benzodiazepines).

1.2.3.1 Sample Adulteration

It is relatively common for drug misusers to attempt to influence their urine drug results (Dasgupta, 2007). For negative results, individuals may drink a large volume of fluid prior to the test in an attempt to dilute the urine, or water may be directly added to the urine sample. Other substances (e.g. bleach, detergents, salt) may be added to the urine sample in an attempt to interfere with the laboratory tests (Jaffee *et al.*, 2007). Conversely, samples may be adulterated to give positive results through direct addition of drugs to appear as if individuals are taking prescribed medication, e.g. methadone.

The laboratory may be able to identify cases of adulteration through a number of measures (Table 1.9). Low creatinine and specific gravity values may indicate dilute urine. However, a dilute sample does not necessarily indicate that the urine was intentionally diluted (Chaturvedi *et al.*, 2013; Holden and Guice, 2014). The complete absence of creatinine is indicative of a specimen not being consistent with human urine (e.g. tea, orange squash, water).

Creatinine measurement is also useful for comparing drug use in one individual over a period of time as drug concentrations can be normalised to creatinine to account for differences in hydration at the time of sampling. This is particularly relevant for assessing whether the re-use of drugs (most notably cannabis due to its high lipophilicity) is likely (Smith *et al.*, 2009).

Table 1.9 – Urine validity tests and possible causes for increased/decreased values

[a] Taken from Dasgupta, 2003

Validity Test	Normal Range[a]	Cause for decrease	Cause for increase
Creatinine	>1.8 mmol/L	Direct dilution of urine (e.g. addition of water), excessive fluid intake	Exercise, creatine ingestion
pH	4.0-10.0	Addition of acidic chemical (e.g. vinegar, lemon juice)	Addition of basic chemical (e.g. bleach)
Temperature (measured at collection)	32.5-37.2 ^{o}C	Cold urine suggests the sample has not been recently collected	-
Specific gravity	1.005-1.030	Renal failure, diabetes insipidus, excessive fluid intake	Addition of salt, glycosuria, dehydration

The laboratory may also identify cases of adulteration through measuring parent drug concentrations, in particular assessing the parent drug-to-metabolite ratio. Selective methodology is required to measure metabolites, typically using chromatography. Immunoassays are usually unable to differentiate between parent drug and metabolites due to their structural similarities. Some immunoassay manufacturers have tried to overcome this by targeting metabolites, e.g. 2-ethylidene-1,5-dimethyl-3,3-diphenyl-pyrrolidine (EDDP) as opposed to methadone, in separate assays.

Assays have also been developed to attempt to identify intravenous (IV) abuse of disaccharide-containing formulations of buprenorphine and methadone through detection of sucrose and lactose in urine (Jungen *et al.*, 2013, 2017). When taken orally, disaccharidases present in the small intestine break down the disaccharides into monosaccharides. However, when injected intravenously disaccharides are excreted unchanged in urine.

1.2.3.2 Therapeutic Drug Monitoring

Therapeutic drug monitoring (TDM) of drugs used in relapse prevention, maintenance, or withdrawal treatment in substance-related disorders is not established in clinical practice. In order to assess whether TDM may be beneficial, assays to measure the drug (and relevant metabolites) need to be developed and data from a varied population collected to establish if there is a correlation between plasma concentration and effect (either efficacy or toxicity). TDM has been suggested for buprenorphine, methadone and naltrexone (Brünen *et al.*, 2011), although there is much debate over whether it

would be beneficial. Additional problems may arise due to difficulty in obtaining a blood sample from an IV drug user, and handling of higher risk samples due to a greater prevalence of blood-borne diseases.

1.2.3.2.1 Methadone and Buprenorphine

Both methadone and buprenorphine exhibit significant inter-individual variation in metabolism, much of which is due to individual variability in cytochrome P450 (CYP) enzymes, and methadone particularly can be a difficult drug to administer safely requiring initial dose titration (Tetrault and Fiellin, 2012). TDM may be beneficial in the first days of therapy to establish an effective dose in individual patients, and also when an individual is switched from methadone to buprenorphine to help decide the exact schedule of methadone dose reduction and buprenorphine escalation (Mercolini *et al.*, 2007).

Lower plasma total methadone concentrations have been associated with greater likelihood of abuse of non-prescribed opioids (Wolff *et al.*, 1996). Minimum plasma concentrations for methadone of 100-400 µg/L have been reported to be essential for adequate maintenance dosage (Holmstrand *et al.*, 1978; Loimer and Schmid, 1992), and for buprenorphine of at least 0.8 µg/L to avoid opioid withdrawal symptoms (Kuhlman *et al.*, 1998).

Currently, it is deemed that the monitoring of adherence to methadone or buprenorphine, and the efficacy of treatment (judged by abstinence from other opioids) can best be achieved through routine urinalysis.

1.2.3.2.2 Naltrexone

The effectiveness of naltrexone therapy in alcohol dependency is well documented (Goh and Morgan, 2017; Rösner *et al.*, 2010). However, large-scale studies have not been evaluated to the same extent for opioid dependency particularly for extended-release formulations of naltrexone, and there are data lacking to assess correlation of naltrexone plasma concentration and clinical response. These data may provide justification for TDM of naltrexone to aid individualisation of dosing regimens to improve the outcome of naltrexone treatment. A target naltrexone plasma concentration of 1 µg/L has been suggested for blocking a clinically relevant dose (e.g. 25 mg) of intravenously administered diamorphine (Kunøe *et al.*, 2014). As well as providing information to maximise efficacy of naltrexone treatment, TDM may also be beneficial in minimising toxicity. A correlation between urinary 6-β-naltrexol (naltrexone's main plasma

metabolite) concentration and side effects (e.g. headache, nausea, anxiety) has been reported (King *et al.*, 1997). Reported concentrations of plasma naltrexone and 6-β-naltrexol are variable (Table 1.10). To date, a large-scale study to ascertain plasma naltrexone concentrations achieved during therapy for opioid addiction has not been performed. A pilot study conducted by Jarvis *et al.* (2016) looking at factors associated with using opiates whilst under treatment with extended-release naltrexone concluded that future research should incorporate measurement of plasma naltrexone concentration.

Table 1.10 – Plasma naltrexone and 6-β-naltrexol concentrations after oral administration of naltrexone

Study	N	Dose (mg)	Time since last dose (h)	Naltrexone (µg/L)	6-β-Naltrexol (µg/L)
Huang *et al.* (1997)	5	25	5.5	1.4-9.9	13.7-29.7
Huang *et al.* (1997)	3	25	27.5	0.1-0.4	-
Huang *et al.* (1997)	5	25	125.5	-	0.3-0.6
Heinälä *et al.* (2012)	87	50	2-4	0-70	15-136
Verebey *et al.* (1976)	4	100	4	8.9-32.4	49.3-85.1
Huang *et al.* (1997)	5	100	5.5	6-27	57.4-106.8
Huang *et al.* (1997)	5	100	27.5	0.4-0.9	-
Huang *et al.* (1997)	5	100	125.5	-	0.8-2.1
Verebey *et al.* (1980)	4	400	16	4.8-20.4	143.9-251.8

1.2.3.3 Pharmacokinetic Studies

Assays may be developed for the measurement of drugs in a research context, primarily for pharmacokinetic studies. In terms of drugs of abuse, this may be looking at different routes of drug administration, or comparison of drug formulations (e.g. extended release versus immediate release) to improve clinical outcomes.

1.2.4 Analytical Methods

Many analytical methods may be used for drug analysis in biological samples, and depending on the clinical requirements may yield qualitative or quantitative results. The choice of methodology will often depend on the chemical properties of the analyte, required turn-around-time (i.e. the time from sample receipt to reporting results), and the cost of the test. The most crucial aspect of any method is that it must produce accurate, reliable and reproducible results. To assess this, any new method developed must undergo full method validation before it is used routinely. Validation of qualitative

methods does not need to be as comprehensive as that for a quantitative method, but should include tests for ascertaining selectivity, precision, matrix effect, recovery, detection limits, analyte carryover, and stability (Trullols *et al.*, 2004). When developing a method, both the pre- and post- analytical stages must also be considered. Of particular importance is ensuring the sample is collected, transported and stored in a suitable manner (Flanagan, 2004).

1.2.4.1 Immunoassay

Immunoassays offer the benefit of speed, high throughput, and ease of automation. For this reason, immunoassays have typically been used for initial drug screening purposes (e.g. amfetamine and opioid group assays). However, the utility of immunoassays is often limited by their low specificity. For example, a positive opioid result may not aid clinical management of a patient as use of an over-the-counter (OTC) medication (e.g. codeine) cannot be distinguished from illicit drug use (e.g. heroin). The clinical picture may also be confused by false positive results occurring from immunoassays, for example amfetamine assays are prone to interference from many prescribed drugs (Table 1.11). The necessity of confirming immunoassay-positive results through use of more specific techniques, usually chromatography-based, causes an increase in turn-around-time and laboratory expenditure.

In addition, only a fraction of toxicologically relevant substances can be covered by currently available immunoassay techniques. Many important analytes (e.g. gamma-hydroxybutyrate (GHB)) do not have commercially available immunoassays.

The cross-reactivity and ability of immunoassays to detect NPS is currently unclear. Stimulant-type NPS are often structurally related to amfetamine or MDMA. However amfetamine-group immunoassays often may not have sufficient cross-reactivity to these novel compounds and thus drug use may go undetected (Beck *et al.*, 2014). Similarly, synthetic cannabinoids are unlikely to cross-react with cannabis immunoassays where antibodies are typically raised against THC derivatives due to the significant structural differences in these novel compounds from THC itself. Immunoassays for detecting synthetic cannabinoids are being marketed, with the majority to date targeting JWH-018 and structurally-related cannabinoids (Barnes *et al.*, 2014, 2015). However, due to the time and expense in developing an immunoassay it is likely that when the assay becomes available it will no longer be suitable as current NPS will have changed and thus may exhibit minimal cross-reactivity, meaning their use goes undetected.

Table 1.11 – Reported interferences in urine immunoassays

Immunoassay	Interference	Reference
Amfetamine	Dimethylamylamine (DMAA)	Vorce *et al.* (2011)
	Labetalol (via the metabolite 3-amino-1-phenylbutane)	Yee and Wu (2011)
	Mebeverine	Kraemer *et al.* (2001)
	Bupropion	Casey *et al.* (2011); Vidal and Skripuletz (2007)
	Chlorpromethazine/promethazine	Melanson *et al.* (2006)
	Fenofibrate	Kaplan *et al.* (2012)
	Phentermine	Marin *et al.* (2009)
	Trazodone (via the metabolite *meta*-chlorophenylpiperazine, mCPP)	Logan *et al.* (2010); Roberge *et al.* (2001)
Benzodiazepine	Oxaprozin	Matuch-Hite *et al.* (1995); Nishikawa *et al.* (1999)
Buprenorphine	Opioids (high concentration)	Pavlic *et al.* (2005)
	Amisulpride/sulpiride	Birch *et al.* (2013)
	Tramadol	Shaikh *et al.* (2008)
Cannabis	Efavirenz	Oosthuizen and Laurens (2012); Rossi *et al.* (2006)
Opioid	Gatifloxacin	Straley *et al.* (2006)
	Rifampicin	de Paula *et al.* (1998)

1.2.4.2 Chromatographic Methods

Chromatography is a separation method based on partitioning of compounds, as a result of their physicochemical properties, between a stationary phase and a mobile phase. The mobile phase (e.g. liquid, gas) carries the components of a sample through the stationary phase (e.g. silica) where separation occurs. As a result, an analyte has a characteristic time that it takes to pass through a system (from injector to detector), termed the retention time (RT). A longer RT indicates a stronger interaction between the analyte and stationary phase at constant mobile-phase flow.

Early methods for drug detection used thin layer chromatography (TLC) to provide qualitative information on analytes present in a sample. However, interpretation of TLC plates is highly subjective, the required reagents are often toxic, and data are not very reproducible (Flanagan *et al.*, 2007).

Gas chromatography (GC) has been widely used in analytical toxicology, particularly in conjunction with flame ionisation detection (FID), which provides a universal and

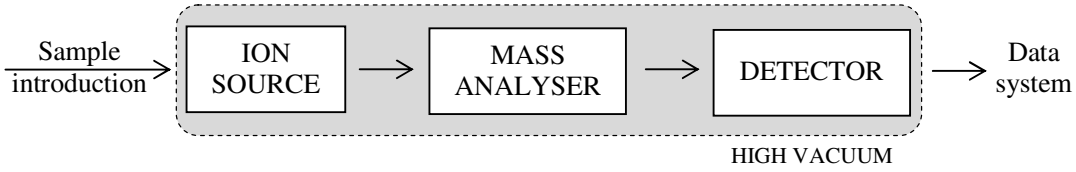
sensitive detection method. Selectivity can be improved through use of electron capture detection (ECD) for halogen-containing compounds (e.g. benzodiazepines), or mass spectrometry (MS). GC remains the gold standard for analysis of volatile compounds, e.g. ethanol, methanol, butane, acetone.

Liquid chromatography (LC) is a more versatile method than GC, and is well suited to analysis of hydrophilic and thermally labile compounds. As a result sample preparation is often simpler than that associated with GC. Numerous LC parameters can be varied to achieve analyte separation including mobile phase composition, column stationary phase chemistry, column temperature, and use of gradient elution. LC can be performed in several modes, including reverse-phase (accounting for the majority of methods), normal phase, hydrophilic interaction chromatography (HILIC), ion exchange, and size exclusion. Reverse-phase chromatography uses a polar eluent (e.g. acetonitrile, methanol) and a non-polar stationary phase surface (typically silica modified by the addition of octadecyl (ODS, C_{18}) or octyl (C_8) silyl moieties. Numerous detection methods may be coupled to LC including ultra-violet (UV) absorbance, fluorescence, and MS.

1.2.4.3 Mass Spectrometry

A mass spectrometer generates ions, and then separates them based upon their mass-to-charge ratio (*m/z*). The basic components of a mass spectrometer include: an inlet device for sample introduction, an ion source, a mass analyser, a detector and a data system (Figure 1.7).

Figure 1.7 – Components of a mass spectrometer



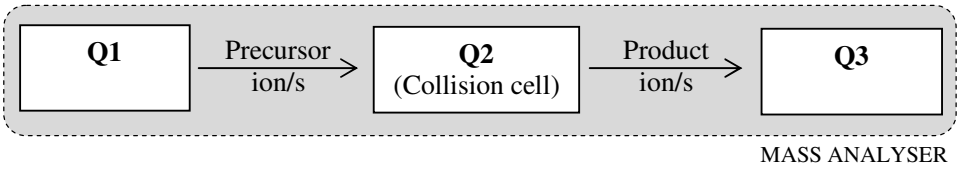
The method of ionisation is largely dependent on how the sample is being introduced. When coupled to LC, the most common methods of ionisation are either electrospray ionisation (ESI) or atmospheric pressure chemical ionisation (APCI). For GC, either electron ionisation (EI) or chemical ionisation (CI) may be used. Other specialist ionisation techniques have also been developed, e.g. matrix-assisted laser desorption ionisation (MALDI). Ionisation methods may be termed 'hard' where the molecules present in a sample are subjected to high energy causing a large degree of

fragmentation, or 'soft' where little energy is applied resulting in minimal fragmentation. EI is the most common example of hard ionisation. Fragmentation that occurs during ionisation is termed 'in-source fragmentation'. Ionisation may be performed in either positive (typically generating $[M+H]^+$ ions) or negative (typically generating $[M-H]^-$ ions) mode.

Many types of mass analyser are available including: quadrupole, time-of-flight (TOF), magnetic sector, and ion trap. Combinations of analysers are also possible, for example quadrupole time-of-flight (Q-TOF). The resulting signal from the mass analyser is converted into useful data by the detector, which the analyst can then interpret using an associated data system.

Analyte detection may be based either on the precursor ion, or on product ions measured by the mass analyser. Product ions are formed when using tandem mass spectrometry (MS/MS, MS^2), which involves selection and fragmentation of a precursor ion and measurement of one or more product ions. Typically quantitative methods have been performed on triple quadrupole instruments (QQQ) using selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) where one or more precursor ions are selected in the first quadrupole, fragmented in a collision cell, and then one or more product ions are selected in the final quadrupole for detection (Figure 1.8). Qualitative, or screening, methods are more often performed on scanning instruments (e.g. TOF, ion traps) where data are collected for all ions within a specified *m/z* range and utilises the presence of precursor ions for analyte detection and identification. A more selective experiment for monitoring parent ions is selected ion monitoring (SIM), where data are only collected for the *m/z* ratios of interest, which may be more applicable to targeted methods. Different scan experiments may all be included in a single method, but increasing the number of experiments will lead to a reduction in the number of scans over a chromatographic peak.

Figure 1.8 – Schematic of selection reaction monitoring by a triple quadrupole mass spectrometer



Gas chromatography-mass spectrometry (GC-MS) has long been regarded as the 'gold standard' for unknown drug screening ('systematic toxicological analysis', STA), particularly for forensic work. The main advantage of GC-MS is that most instruments use standardised MS conditions, meaning that mass spectra of the same compound measured by different analysts on different instruments produce the same results. As a result, universal mass spectral libraries based on EI data can be reliably searched to aid compound identification. However, GC-MS has the disadvantage of time-consuming sample preparation steps, such as derivatisation, which is often not suitable for clinical samples where a fast turnaround of results is required for patient treatment. There are other known pitfalls associated with GC-MS such as degradation of thermally labile compounds leading to artefacts, for example conversion of methadone to its metabolite EDDP (Galloway and Bellet, 1999).

Due to its increased versatility, development of LC-MS (and LC-MS/MS) methods for clinical drug detection is becoming a preferable alternative to immunoassay and GC-MS methods. However, searchable databases are still lacking compared to those available for GC-MS, mainly due to the lack of reproducibility of collision-induced dissociation (CID) spectra, which are sensitive to instrument design and operational parameters such as ionisation source settings, eluent composition, flow rate, and the presence of organic eluent modifiers (Hough *et al.*, 2000; Josephs and Sanders, 2004). LC-MS(/MS) methods are associated with high specificity, particularly tandem MS methods, although pre-selection of analytes is required and thus the analysis is targeted. Collection of full-scan data, where all precursor ions within a selected *m/z* range are detected, avoids the targeting limitations of MS/MS, but can mean that selectivity is reduced due to the presence of background noise (Jagerdeo and Schaff, 2016). High-resolution mass spectrometry (HRMS) can often overcome this issue by allowing an analyte signal to be extracted from a near-isobaric background signal.

1.2.4.4 High Resolution Mass Spectrometry

MS resolving power is the ability of a mass spectrometer to separate ions with different *m/z* values, and is calculated using the width of mass spectral peaks at half their maximum height (full width half maximum, FWHM, Figure 1.9). The International Union of Pure and Applied Chemistry (IUPAC) definition (Murray *et al.*, 2013) for resolution in mass spectrometry is given below (Equation 1.1).

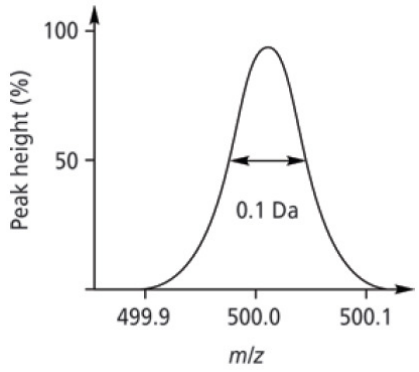
Equation 1.1 – IUPAC definition of resolution

$$Resolution\ (R) = \frac{mass\ of\ the\ ion\ of\ interest\ (m/z)}{peak\ width\ (FWHM)}$$

Resolution is *m/z* dependent, and the measured, or observed, resolution will therefore vary across an acquired mass spectrum which covers a large *m/z* range. The observed resolution, and the observed variation in resolution as a function of *m/z*, is dependent on the mass analyser used.

High MS resolving power is necessary to separate adjacent peaks from one another and to ensure that only one ion contributes to the measurement, thus enhancing selectivity. Mass accuracy (typically expressed in parts per million, ppm) determines how close the measured (observed) *m/z* is to the theoretical *m/z* (Equation 1.2). The terms "high resolution" and "high mass accuracy" (or "accurate mass") are commonly used when resolution >20,000 FWHM and mass accuracy below 5 ppm, respectively, are achieved (Balogh, 2004). High resolution in conjunction with accurate mass measurements enables the elemental composition of a compound to be ascertained, which has particular importance for identification of unknown substances in a drug screen.

Figure 1.9 – FWHM method for determining resolution for a mass spectrometer measured at a given ion (taken from Balogh, 2004)



Mass = 500
Peak width (at 50%) = 0.1
Resolution (FWHM) $\frac{500}{0.1} = 5000$

Equation 1.2 – Calculation of mass accuracy

$$Mass\ accuracy\ (ppm) = \frac{measured\ mass - theoretical\ mass}{theoretical\ mass} \times 10^6$$

Within clinical laboratories, HRMS instruments are either TOF or orbitrap (OT) instruments. TOF instruments measure ion *m/z* based on the measurement of elapsed time from the pusher plate to the detector, whereas OT instruments measure *m/z* by the frequency of ion oscillation in a magnetic and electric field. TOF instruments have the advantage of maintaining rapid scanning rates at high mass resolution, unlike OT where resolution is directly proportional to the acquisition time, i.e. the longer the acquisition time the higher the resolving power. TOF instruments also have a higher upper *m/z* limit; however as the majority of drugs are small molecules this is not a significant advantage in the context of toxicological analyses. Slower MS scan rates can lead to reduction of data points across a chromatographic peak, thus it is important that increasing the MS resolution does not comprise the integrity of a chromatographic peak, where at least 10 data points are required for reliable quantitation (Holčapek *et al.*, 2012). However, TOF instruments are more prone to environmental changes (temperature, humidity) and as a result must be situated in a tightly regulated environment to maintain mass stability (Ojanperä *et al.*, 2012). More modern TOF instruments have design modifications to overcome these issues, e.g. super-insulated flight tubes.

High-resolution instruments are usually operated in an untargeted manner, which means that accurate mass information is collected on every ion that reaches the detector. This is advantageous for several reasons, but most importantly, it allows for the detection and identification of compounds that are unexpected, e.g. newly emerging NPS. However, complications of detecting NPS arise through i) a lack of understanding of metabolic profiles and hence which compounds will be present in different biological sample matrices, ii) a lack of reference materials, and iii) low analyte concentration due to the high potency of some NPS, e.g. synthetic cannabinoids.

While much of the interest in LC-HRMS revolves around identifying unexpected compounds, the vast majority of samples submitted for urine drug screening will contain common drugs. HRMS may be a promising approach in a clinical situation where a combination of unknown screening for novel compounds and targeted screening for well-established drugs of abuse is required (Maurer and Meyer, 2016). HRMS data may be processed for specific analytes appropriate for a routine urine drug screen, but in addition the full data for a sample are collected and unknown screening can be conducted manually in retrospect. The advantages and disadvantages of using LC-HRMS for urine drug screening are summarised in Table 1.12.

Table 1.12 – Advantages and disadvantages of LC-HRMS as compared to targeted LC-MS/MS methods

Advantages	Disadvantages
• Untargeted compounds may be identified • Retrospective analysis of data is possible • Method development is simpler and faster • Flexibility to add novel drugs/metabolites quickly to existing screening methods	• Loss of sensitivity as a result of full scan data collection • Data analysis may be complex due to the vast amount of data collected on each sample • HRMS instruments are more expensive • Data evaluation software is insufficient for broad screening and requires skilled operators

1.3 Aims and Objectives of This Work

To explore the role of the laboratory in routine screening for substance misuse, with the aim of improving services to give clinicians more information regarding their patient's drug use. A novel method using LC-HRMS will be developed and validated for this purpose. Application of this method for routine urine drug screening and identification of unknown substances will be explored.

To audit the clinical data resulting from routine urine drug screens over a period of one year with the aim of establishing current trends of drug use from the surrounding population. In addition, the data will be interrogated to identify any pitfalls of drug testing (e.g. sample adulteration) and demonstrate how the laboratory may be able to identify these problems and aid clinicians in overcoming them.

To explore how the laboratory can assist with new approaches to drug treatment strategies. A novel LC-HRMS method will be developed and validated with the purpose of performing pharmacokinetic studies on opioid antagonists administered via alternative administration routes, such as buccal.

2 Qualitative Drug Analysis by LC-HRMS: Application to routine clinical screening for drugs in urine samples

2.1 Introduction

As described in Chapter 1, LC-HRMS may be a promising approach for routine drug screening in a clinical situation where a combination of unknown screening for novel compounds and targeted screening for well-established drugs of abuse is required. The framework currently used by many laboratories consists of initial immunoassay screening followed by confirmatory chromatographic methods. This approach is poorly suited to laboratories where most samples are positive for at least one analyte, which results in most samples undergoing duplicate testing. Despite a high initial capital outlay, the use of LC-HRMS for combined screening and confirmation of drugs of abuse may result in considerable time and long-term financial savings. In addition, less ambiguous results are generated through detection of specific analytes as opposed to drug classes, and there is the potential to detect a wide array of drugs, metabolites and adulterants in a single analysis.

Many LC-HRMS drug screening methods have been published including screening for cardiovascular drugs (Helfer *et al.*, 2015), NPS (Concheiro *et al.*, 2015; Montesano *et al*., 2016; Sundström *et al.*, 2013), diuretics (Girón *et al.*, 2012), and for several classes of drugs of abuse (Li *et al.*, 2013; Marin *et al.*, 2012). Most published methods were developed for forensic as opposed to clinical analysis. One of the challenges posed by clinical analysis is that sample throughput is greater and turn-around-times often need to be faster than in forensic work. It is therefore important that the methods used are robust in terms of scope, sample throughput and ease of operation to be useful in clinical as opposed to forensic laboratories (Sundström *et al.*, 2013).

One of the current limitations associated with routine clinical use of HRMS for drug screening is the lack of software that easily and unambiguously identifies analytes of interest (Meyer, 2013). Without such software, non-targeted analysis is based on experienced analysts visually recognising large peaks in the total ion chromatogram (TIC) and ascertaining molecular formula matches (Meyer and Maurer, 2016). Further investigation of possible molecular formulae is necessitated, requiring internet and literature searches to enable putative identification. Due to the huge quantities of data generated for each sample, manual processing is not feasible for routine services and is in the main limited to research work or one-off assay requests.

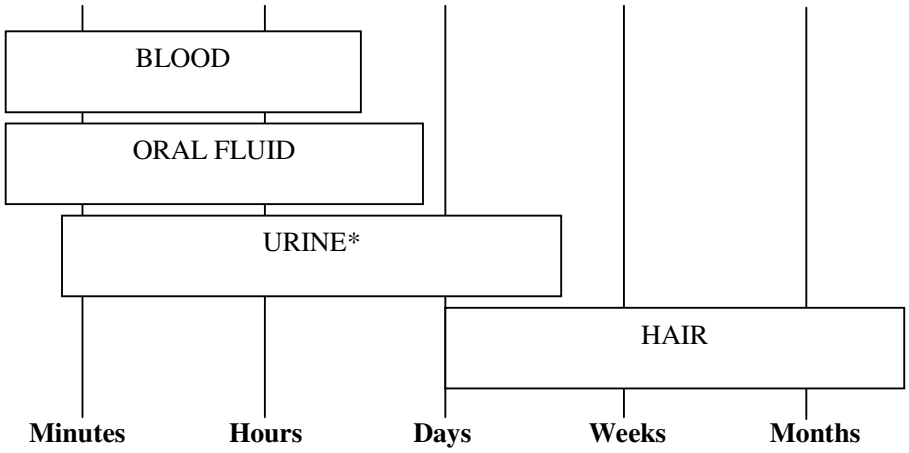
The use of accurate mass measurements (accurate to 3 or 4 decimal places rather than nominal or unit mass measurements of +0.1 amu) aids compound identification through significantly reducing the number of compounds potentially giving rise to a peak. However, there are a large number of isobaric drugs and metabolites that can hinder analyte identification (Guale *et al.*, 2013). Comparison of a measured accurate mass to a theoretical exact mass can therefore only provide a screening result that needs further confirmation. Additional confirmatory parameters such as relative retention time (to an internal standard), specific product ions, and detection of metabolites (where possible) markedly increases the reliability of analyte identification. Identification of putative metabolites through accurate mass alone is not sufficient as metabolites of isomeric compounds may have the same accurate mass as a result of a common metabolic process, e.g. demethylation (loss of a methylene moiety). By using multiple confirmatory parameters, the reliability of compound identification is comparable to a targeted analysis.

2.1.1 Biological Matrices

The selection of a suitable biological matrix to detect drug use is largely dependent on the timeframe of drug use that is of interest. The detection window for drug exposure varies according to which sample is used in the analysis (Figure 2.1).

<u>Figure 2.1</u> – Typical detection windows for drugs of abuse in various common biological samples

*with the exception of cannabis which may be detected up to a month after cessation in chronic users



Urine is the most commonly used matrix for monitoring drug use in individuals undergoing drug rehabilitation therapy. This is largely as collection is non-invasive and yields a large sample volume with relatively high drug and metabolite concentration. In addition, analytically urine offers the benefit of requiring minimal sample preparation as protein should not be present in the sample. A disadvantage associated with urine collection for drugs of abuse measurement is that samples may be easily adulterated in an attempt to mask drug-taking behaviour.

Drugs of abuse may be measured in blood (typically plasma or serum), but this is usually only of benefit when quantitation is required to assess impairment. Obtaining blood from an intravenous drug user can also prove challenging. Oral fluid has been advocated as an alternative matrix to urine for screening for drugs of abuse (Bosker and Huestis, 2009; Vindenes *et al.*, 2011). Drug concentrations in oral fluid are said to reflect the free, unbound parent drug and lipophilic metabolites circulating in the blood. Oral fluid has the benefit of being a non-invasive and non-private sample, and samples are more difficult for users to adulterate. However, analytically oral fluid is a more complex matrix than urine and analyte concentration may be very low, especially for acidic drugs which do not readily partition into oral fluid (Cone and Huestis, 2007). Problems may also be encountered in regard to adsorption to collection devices, particularly for lipophilic drugs, giving low analyte recovery (Bosker and Huestis, 2009).

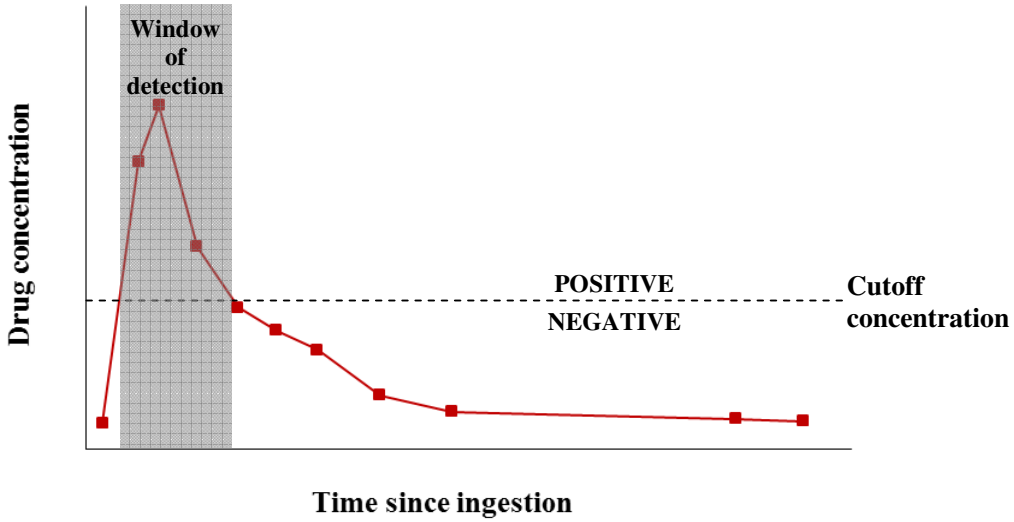
Hair analysis offers the possibility of longer detection times when compared to other matrices, and may offer a means of evaluating an individual's drug use history. Hair analysis is rarely conducted for clinical purposes, and is more often used in workplace drug testing and in forensic or legal cases (e.g. drug-facilitated crimes) (Xiang *et al.*, 2015). There are however problems with hair analysis, particularly concerning external contamination, cosmetic treatments, ethnical bias and the rate of drug incorporation (Kintz *et al.*, 2006).

2.1.2 Qualitative Measurement of Drugs

In urine drug testing, most results are reported as positive (present) or negative (absent) for a substance based upon a cutoff concentration (Figure 2.2). The cutoff concentration selected is based upon optimising drug detection but minimising the number of false positive results, and largely originates from the use of immunoassay for drug screening. The selected cutoff for an immunoassay test is dictated by the manufacturer. Cutoff

concentrations are also used for confirmatory drug analyses, and may be lower than screening cutoffs due to the more specific methodology used (see Section 2.3.4 for further information). When using selective methodology such as LC-HRMS, it may be more appropriate to use the limit of analytical detection as opposed to the cutoff concentration for certain cases (e.g. samples from children, suspected drug-facilitated assault).

Figure 2.2 – Visual representation of a cutoff concentration for drug detection in urine



2.1.3 Sample Preparation

The aim of sample preparation is to improve assay sensitivity/selectivity and remove compounds present in the sample matrix that may interfere in the analysis (Dams *et al.*, 2003; Peters and Remane, 2012). A 'matrix effect' is defined as the effect of other compounds in the sample matrix on the quantitation of the analyte. In LC-MS, endogenous or other compounds that co-elute with an analyte may either suppress or enhance ionisation of an analyte in the source of the mass spectrometer. As the presence of these substances may vary between samples it is important that internal standards are used to compensate if possible for any variation in analyte ionisation to ensure accurate quantitation. Regardless of the preparation technique used, an internal standard should be added at the beginning of the process to account for any variation during extraction/dilution.

Many modes of sample preparation exist. Selection of an appropriate method will often depend on the required level of sensitivity, the chemical properties of the analyte(s), the number of analytes, and the matrix being analysed.

For drug analysis, the most commonly used selective sample preparation techniques are liquid-liquid extraction (LLE) and solid-phase extraction (SPE). LLE involves extraction of an analyte from a biological matrix into an immiscible organic solvent and is based upon the partition coefficient of the analyte between the matrix and the solvent. For ionisable compounds, typically, the extraction pH should be 2 units more than the pK_a for basic analytes and 2 units less than the pK_a for acidic analytes. Under these conditions analytes will be <1 % ionised and hence partition into the organic solvent will be favoured (Flanagan *et al.*, 2007). The extraction pH may need to be a compromise when several different compounds are being assayed. For drug analysis, LLE is not suitable for extracting glucuronide metabolites due to their hydrophilic nature.

SPE involves analyte absorption onto a solid phase, commonly with reverse-phase chemistry (e.g. C_8, C_{18}). To capture a broader range of compounds 'mixed-mode' SPE columns may be used which utilise two or more retention mechanisms. As with LLE, SPE has the benefit of allowing analyte concentration in an extract, which may be beneficial for compounds present at low concentration in samples. SPE is more compatible with automation than LLE, and thus has advantages for high-throughput batch analysis of samples.

TurboFlow technology (ThermoFisher Scientific), a form of SPE, is a column-based on-line sample preparation system that utilises the principles of size-exclusion chromatography and specific column-based chemistry. TurboFlow enables the direct injection of biological samples allowing sample preparation to be simplified and throughput increased (Couchman, 2012). Sample is loaded onto the TurboFlow column under aqueous conditions allowing the analyte to bind to the column packing whilst proteins and other matrix components flow to waste. The analytes are then eluted from the TurboFlow column, and transferred to the analytical LC column. TurboFlow offers the benefit of automated sample preparation, which minimises human error thus making methods more robust, and also reduces the time required to perform an assay.

Dilution is a non-selective technique which may be a beneficial approach for analysing a wide range of analytes. However, it is likely that matrix effects will be higher than if a selective method is used. For matrices containing low protein concentration (e.g. urine), centrifugation followed by dilution may be satisfactory. For matrices containing protein (e.g. whole blood, plasma), protein precipitation using acid (e.g. trichloroacetic) or an organic solvent (e.g. acetonitrile) may be used for non-selective sample preparation.

2.1.3.1 Urine Hydrolysis

Many drugs (e.g. opioids, benzodiazepines) are predominantly excreted in urine as conjugates, typically with D-glucuronic acid or with sulfate. As a result, urine samples may be hydrolysed prior to analysis to convert these conjugated metabolites to the unconjugated analyte. Hydrolysis may be non-specific through use of an acid (e.g. hydrochloric acid). Acid hydrolysis is very effective, but the resultant solutions may corrode metal instrument components resulting in increased maintenance frequency and cost. Acid hydrolysis may also destroy specific metabolites, e.g. 6-AM, complicating interpretation of results (Sitasuwan *et al.*, 2016; Zezulak *et al.*, 1993). As a result most laboratories perform selective hydrolysis using an enzyme solution, typically β-glucuronidase for glucuronide analysis. Hydrolysis is needed for sensitive GC-MS analysis, but is not required when LC-MS is used as direct analysis of polar conjugates is possible (Peters, 2011). LC-MS methods which use a hydrolysis step must ensure that solubilised β-glucuronidase is removed from the solution before HPLC analysis. This is because if β-glucuronidase is not removed the column lifetime will be shortened due to enzyme precipitating in the column and causing increased back pressure. Whilst sensitivity may be improved through hydrolysis, information regarding metabolism and time since drug intake is lost, information that may prove useful when interpreting results.

When hydrolysis is used, drug concentrations are reported as a 'total'. For example, 'total morphine' reflects both the unconjugated 'free' morphine in a urine sample and morphine produced as a result of hydrolysis of morphine-3-glucuronide and morphine-6-glucuronide. Where hydrolysis is not used, 'total' drug concentrations can be calculated (Equation 2.1) by converting conjugated metabolites to the 'free' drug equivalent (assumes complete conversion to 'free' drug).

<u>Equation 2.1</u> – Conversion of conjugate metabolite concentration to 'free' drug equivalent concentration

$$\text{'Free' drug equivalent concentration } (\mu g/L) = \text{Conjugate concentration } (\mu g/L) \times \frac{\text{Molecular weight 'free' drug}}{\text{Molecular weight conjugate}}$$

2.1.4 Analytes in a Routine Drug Screen

A wide range of drugs are misused, including well-established drugs of abuse (e.g. heroin, cocaine, cannabis, metamfetamine, MDMA), prescription medication (e.g. buprenorphine, oxycodone, methadone, methylphenidate, pregabalin), and NPS. Ideally a routine drug screen will detect as many of these compounds as possible; however, the majority of clinical drug screening methods currently only target well-established drugs of abuse and methadone/buprenorphine.

As well as targeting parent drugs in a routine screening method, inclusion of metabolites is important for detection of sample adulteration and for providing information on the time since drug administration. Metabolite measurement may also be used in place of detecting the parent drug when the plasma half-life of a drug is very short (e.g. cocaine use may be identified through detection of its major metabolite, benzoylecgonine).

Clinical management of individuals poisoned with some common drugs of abuse is well defined. Whilst treatment of poisoning is typically symptom-directed supportive care, identification of which substances are present can guide treatment of an individual in certain scenarios. Rapidly detecting fetal exposure to licit and illicit drugs is of considerable medical value. It is particularly relevant for diagnosing neonatal abstinence syndrome (NAS), a post-natal opioid withdrawal that can occur in a newborn whose mother was dependent on opioids whilst pregnant, and where the neonate may require OST.

Psychosis can be associated with acute recreational drug toxicity. However, there are limited data available on how common this is and which drugs are most frequently implicated (Vallersnes *et al.*, 2016). Psychosis may also be a result of chronic drug use, with epidemiological evidence demonstrating that regular or heavy cannabis use increases the risk of developing psychotic disorders (Gage *et al.*, 2016). Synthetic cannabinoids and high potency cannabis (containing high THC concentration relative to cannabidiol) are thought to pose the greatest risk (Murray *et al.*, 2016). Accurate detection of such drugs may aid clinical differentiation of the likely cause of a psychosis.

The pharmacology of NPS is poorly understood and clinical management is complicated by the associated unpredictable effects. A major health problem arises from that the fact that mixtures are inhomogeneous with regard to their active ingredients,

particularly with synthetic cannabinoids (World Health Organisation, 2014a). Publications are limited to case reports, and no large randomised controlled trials to assess population pharmacokinetics and toxicity have been conducted. Establishing accurate data on the prevalence, use and effects of NPS remains crucial to establishing successful management of the problems associated with these drugs. In the absence of rapid urine or blood tests to confirm the use of NPS assessment has to be clinical, based on patient history if available, and recognising the clinical presentation, which to date is not well documented.

2.1.5 Aims

To develop a LC-HRMS method capable of detecting and identifying commonly encountered drugs in urine from substance abusers. The method will ideally replace the two-step methodology of immunoassay followed by LC-MS. The method will be developed so that it is adaptable to include other analytes, e.g. NPS.

2.1.6 Ethics Considerations

Patient samples used for method development will be excess samples collected for clinical purposes and submitted to the laboratory for urine drug screening. No records kept during method development will permit identification of patients.

2.2 Materials and Methods

2.2.1 Chemicals and Reagents

Analyte reference standards and the majority of internal standards were supplied as solutions (either in methanol or acetonitrile) from Cerilliant (Sigma, Poole UK). Amfetamine-$^{13}C_6$ hydrochloride was from Chiron (Trondheim, Norway). Mephedrone-D_3 hydrochloride and norketamine-$^{13}C_6$ hydrochloride powder were from Alsachim (Illkirch-Graffenstaden, France). External quality control (EQC) samples were from ACQ Science GmbH (Rottenburg am Neckar, Germany).

HPLC grade acetonitrile, ammonium formate and formic acid were from Sigma-Aldrich (Poole, UK). HPLC grade methanol was from Rathburn (Walkerburn, UK). Water was deionized (18 mΩ, Elga, Marlow, UK). Positive and negative ion mass calibration solutions were from ThermoFisher Scientific. Analyte-free human urine was obtained from a volunteer. HPLC vials and caps were from Kinesis (St. Neots, UK), and 0.5 mL polypropylene tubes were from Sarstedt (Nümbrecht, Germany). DRI creatinine, CEDIA barbiturate, benzodiazepine, cannabis, cocaine, methadone metabolite, and buprenorphine assays, calibrators, and IQCs were from ThermoFisher Scientific (Microgenics, Passau, Germany). Liquichek™ urine chemistry controls (creatinine IQC) were from Bio-Rad (Watford, UK).

2.2.2 Instrumentation

An automated dilutor was used for sample preparation (Hamilton MicroLab 530 C, Esslab, UK). An Ultimate 3000 Dionex HPLC system (ThermoFisher Scientific) consisting of two pumps, autosampler and column oven compartment was used with a Q Exactive™ mass spectrometer (ThermoFisher Scientific). Instrument control was performed using TraceFinder™ software (version 3.3, ThermoFisher Scientific).
For all post-acquisition data processing, peak areas were generated by filtering full-scan data using a mass extraction window of ±10 ppm based on theoretical *m/z* values (external mass calibration carried out on alternate days using positive and negative ion mass calibration solutions).

Immunoassays were performed according to the manufacturer's instructions using an Indiko Plus analyzer (ThermoFisher Scientific). Assays were calibrated as necessary using the calibrators supplied, and supplied internal quality control (IQC) solutions were analysed prior to the analysis of patient samples.

2.2.3 Liquid Chromatography

System eluents were as follows: (A) 10 mmol/L ammonium formate with 0.1 % (v/v) formic acid in deionised water, and (B) 0.1 % (v/v) formic acid in acetonitrile:methanol (1+1). Prepared samples were analysed directly (50 µL) using an Accucore Phenyl-Hexyl HPLC column (2.7 µm aps, 100 x 2.1 mm I.D., ThermoFisher Scientific). The column was fitted with a 0.5 µm pre-column filter (ThermoFisher Scientific) and was maintained at 40 °C. Gradient elution (total flow 0.3 mL/min) was used. In summary, the starting conditions were 98 % A 2 % B held for 1.5 min, then ramped to 100 % B over 8.5 min, held for 3 min, then returned to initial conditions for 2 min to re-equilibrate. During re-equilibration, the flow rate was increased to 0.6 mL/min to improve column washing. The total analysis time was 15 min.

2.2.4 Mass Spectrometry

MS was carried out in positive ionisation mode using heated ESI [spray voltage 4.5 kV; temperatures: vaporiser 350 ^{o}C, capillary 250 ^{o}C; auxiliary, sheath and sweep gases 10, 55, and 0 (arbitrary units, respectively), S-lens voltage 70 V]. Full-scan MS data were acquired using a resolution setting of 70,000 defined as FWHM at *m/z* 200, with a scan range of 100-750 *m/*z (Orbitrap settings: maximum injection time 200 ms, automatic gain control (AGC) 3 x 10^6 ions). Alternate all-ion fragmentation (AIF) MS^2 scans were collected to confirm peak identity following higher-energy collisional dissociation (HCD) in the HCD cell [collision gas nitrogen, stepped normalised collision energy 50 V (±50 %)]. The settings for the MS^2 data were: resolution 17,500, scan range 70-750 *m/*z, maximum injection time 200 ms and AGC target 3 x 10^6 ions.

2.2.5 Assay Calibration and Acceptance Criteria

Calibration standards (N = 3) and a matrix blank (analyte-free human urine) were included at the beginning and end of each batch analysis, with both IQCs included after the first set of calibrators and immediately before the last set of calibrators. Alternate IQC solutions were analysed in singleton after every 10 patient samples throughout the batch. EQC samples were analysed with each batch. Assay acceptance criteria were (i) linear (R^2 >0.98) calibration curves for each analyte, and (ii) at least 67 % (4 out of 6) of IQC samples within 20 % of their respective nominal value.

Peak area ratios (analyte to IS) obtained on analysis of the calibration standards were plotted against concentration to construct calibration graphs. Linear regression intercepts were not forced through zero, and no line weighting was applied.

2.2.6 Preparation of Calibration and Internal Quality Control Solutions

Nominal concentrations for calibration and IQC solutions are given in Table 2.1. Individual stock solutions for each analyte (100 mg/L) were prepared in methanol. Working solutions were prepared for the amfetamine analytes (amfetamine, metamfetamine, MDMA and mephedrone, all 50 mg/L free base in deionised water) and buprenorphine analytes (buprenorphine, norbuprenorphine, buprenorphine glucuronide and norbuprenorphine glucuronide, all 10 mg/L in methanol). Appropriate volumes of each stock solution or working solution were evaporated to dryness in volumetric flasks under a stream of compressed air (Table 2.2). Appropriate volumes of amfetamine working solution were pipetted into each volumetric flask to avoid loss during the dry-down step before reconstitution with analyte-free human urine. After thorough mixing and equilibration (24 h, 2-8 °C), calibration and IQC solutions were stored in approximately 150 µL portions in 0.5 mL polypropylene tubes at -20 °C until required.

Table 2.1 – Drug screening assay: Nominal concentrations of analytes in calibration and IQC solutions

*Street heroin markers: papaverine, noscapine and 6-acetylcodeine
‡Norbuprenorphine, buprenorphine glucuronide and norbuprenorphine glucuronide
#*N*-Desmethyltramadol and *O*-desmethyltramadol

	Nominal Concentration (µg/L)				
	Low Calibrator	**Cutoff Calibrator**	**High Calibrator**	**Low IQC**	**High IQC**
Amfetamine	100	200	1,000	150	250
Metamfetamine	100	200	1,000	150	250
MDMA	100	200	1,000	150	250
Mephedrone	100	200	1,000	150	250
Benzoylecgonine	75	150	1,000	112.5	187.5
Morphine	150	300	1,000	225	375
Morphine-3-glucuronide	150	300	1,000	225	375
Codeine	150	300	1,000	225	375
Codeine glucuronide	150	300	1,000	225	375
Dihydrocodeine	150	300	1,000	225	375
Pholcodine	150	300	1,000	225	375
6-AM	5	10	50	7.5	12.5
Street heroin markers*	5	10	50	7.5	12.5
Methadone & EDDP	125	250	1,000	187.5	312.5
Buprenorphine & metabolites‡	2.5	5	50	3.75	6.25
Ketamine & norketamine	25	50	100	37.5	62.5
Tramadol & metabolites#	100	200	1,000	150	250

Table 2.2 – Drug screening assay: Preparation of calibrator and IQC solutions (final volume calibrators 50 mL, IQCs 100 mL; human urine)

*each analyte at the specified concentration, # added after the drying down step

	Working solution concentration (mg/L)	Volume of working solution added (µL)				
		Low Calibrator	Cutoff Calibrator	High Calibrator	Low IQC	High IQC
Amfetamine#	50*	100	200	1000	300	500
Metamfetamine#						
MDMA#						
Mephedrone#						
Buprenorphine	10*	12.5	25	250	37.5	62.5
Norbuprenorphine						
Buprenorphine glucuronide						
Norbuprenorphine glucuronide						
	Stock concentration (mg/L)	**Volume of stock solution added (µL)**				
		Low Calibrator	**Cutoff Calibrator**	**High Calibrator**	**Low IQC**	**High IQC**
Benzoylecgonine	100	37.5	75	500	112.5	187.5
Morphine	100	75	150	500	225	375
Morphine-3-glucuronide	100	75	150	500	225	375
Codeine	100	75	150	500	225	375
Codeine-6-glucuronide	100	75	150	500	225	375
Dihydrocodeine	100	75	150	500	225	375
6-AM	10	25	50	500	75	125
Papaverine	10	25	50	500	75	125
Noscapine	10	25	50	500	75	125
6-Acetylcodeine	10	25	50	500	75	125
Methadone	100*	62.5	125	500	187.5	312.5
EDDP						
Ketamine	100*	12.5	25	50	37.5	62.5
Norketamine						
Tramadol	100	50	100	500	150	250
N-Desmethyltramadol	100*	50	100	500	150	250
O-Desmethyltramadol						
Pholcodine	100	75	150	500	225	375

2.2.7 Internal Standard Solution

A working internal standard (IS) solution containing 25 µg/L amfetamine-$^{13}C_6$, metamfetamine-D_{14}, MDMA-D_5, mephedrone-D_3, codeine-D_6, morphine-D_3, dihydrocodeine-D_6, ketamine-D_4, norketamine-$^{13}C_6$, tramadol-^{13}C-D_3; 10 µg/L benzoylecgonine-D_3, methadone-D_3; 5 µg/L norbuprenorphine-D_3, buprenorphine-D_4, and 6-AM-D_3 was prepared by appropriate dilution of individual stock solutions with eluent A. The solution was stored at 2-8 ^{o}C when not in use.

2.2.8 Immunoassay Procedures

Immunoassays were performed according to the manufacturer's instructions using an Indiko Plus analyser (ThermoFisher). All assays were calibrated daily using the supplied calibrators. The supplied IQC solutions were analysed daily prior to the analysis of patient samples.

2.2.9 Sample Preparation

Centrifuged urine samples/calibrators/IQCs (50 µL) were diluted with 450 µL working IS solution using an automated Hamilton dilutor directly into HPLC vials. The vials were capped and transferred to a pre-cooled (10 ^{o}C) autosampler tray.

2.2.10 Method Validation Protocol

The method was validated according to the US Food and Drug Administration (FDA) Centre for Drug Evaluation and Research (CDER) guidance for bioanalytical method validation (FDA/CDER, 2013). Intra- and inter-assay accuracy and precision were measured by replicate analysis (N = 10) of the IQC solutions and cutoff calibrator on the same day and by singlicate analysis on different days (N = 5), respectively. To ensure accuracy of results, past external quality assurance (EQA) samples (LGC Proficiency Testing Drugs of Abuse in Urine Scheme rounds 108-115, stored at -20 ^{o}C since the original analysis and thawed at room temperature prior to re-analysis) were assayed and the results compared to the assigned values. The limit of detection (LoD) was ascertained through successive serial dilution (1+1, v/v, with analyte-free urine) of the low calibrator and was based upon the concentration at which the signal-to-noise ratio was >3, and the variation (relative standard deviation, RSD) was <20 % (N = 5). The mass accuracy for a typical batch was ascertained to ensure masses did not deviate outside a 10 ppm window. To investigate ion suppression, solutions containing all analytes at the relevant cutoff concentration were prepared in (i) analyte-free human urine from 20 independent sources, and (ii) eluent A. Prepared solutions were diluted

(1+9, v/v) with eluent A and analysed. The ratio of the peak area of each analyte to that of the relevant internal standard was compared for each analyte in the presence and absence of matrix. Analyte carryover was assessed through consecutive analysis of a urine sample containing low (L) and high (H) analyte concentration in the order L,L,L,H,H,H,L,L,L (L: cutoff calibrator, H: all analytes 100 mg/L).

2.2.11 Patient Samples

500 urine samples submitted for routine drug analysis, received by the laboratory in September 2015, were analysed using existing immunoassay procedures and the developed LC-HRMS method. Samples were sent to the laboratory to monitor misuse of drugs, adherence to prescribed OST (e.g. methadone), and to aid patient diagnosis (e.g. admission to hospital with drug overdose).

2.3 Results and Discussion

2.3.1 Analyte Selection

The main consideration for the initial selection of analytes to include in the assay was to ensure continuation of a comparable service to that currently offered by the laboratory, i.e. all the major analytes that cross-react significantly with the immunoassays should be included in the LC-HRMS method. Developing the method in accordance with service user requirements was also important, and as a result ketamine and tramadol were added to the test repertoire. At the time of initial method development, mephedrone was one of the most prevalent NPS and thus was included in the LC-HRMS method. No other NPS were included initially, but scope to add analytes was taken into account during method development. A complete list of analytes selected for the initial method development is given in Table 2.3.

Table 2.3 – Drug screening assay: Analytes selected for the initial method development

Drug Class	Specific analytes
Amfetamines	Amfetamine, metamfetamine, MDMA
Barbiturates	Amobarbital, butabarbital, phenobarbital, pentobarbital, secobarbital
Benzodiazepines	Diazepam, temazepam, temazepam glucuronide, oxazepam, oxazepam glucuronide, chlordiazepoxide, demoxepam, lorazepam, lorazepam glucuronide, clonazepam, 7-aminoclonazepam, nitrazepam, 7-aminonitrazepam, alprazolam
Buprenorphine	Buprenorphine, norbuprenorphine, buprenorphine glucuronide, norbuprenorphine glucuronide
Cannabis	11-carboxytetrahydocannabinol (11-COOH-THC, 11-nor-Δ^9-THC-COOH)
Cocaine	Benzoylecgonine
Ketamine	Ketamine, norketamine
Methadone	Methadone, EDDP
NPS	Mephedrone
Opioids	6-AM, morphine, morphine-3-glucuronide, codeine, codeine-6-glucuronide, dihydrocodeine
Street Heroin	6-Acetylcodeine, noscapine, papaverine
Tramadol	Tramadol, *N*-desmethyltramadol, *O*-desmethyltramadol

2.3.2 Liquid Chromatography Method Development

2.3.2.1 Choice of LC Column

The selected analytes have different physicochemical properties meaning that the LC column selected must be suitable for a broad range of compounds; and in particular needs to be capable of retaining polar metabolites such as glucuronides. Most published methods for drug screening have used reverse-phase chromatography with gradient elution (Table 2.4).

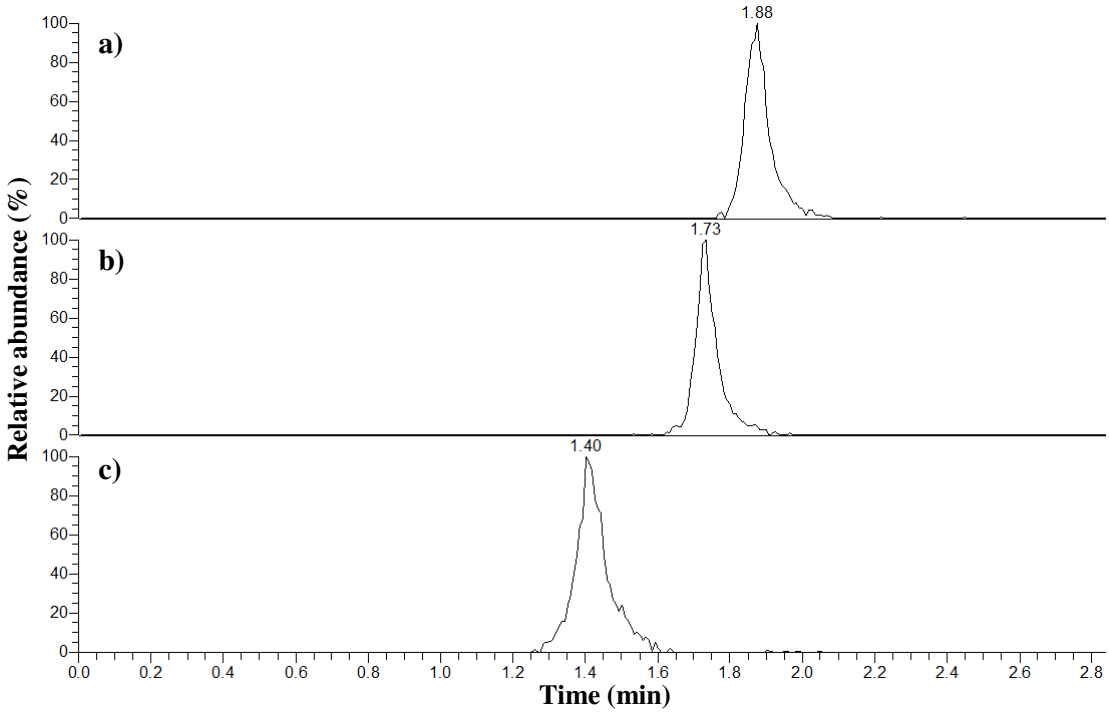
Table 2.4 – Some published LC methods for screening for drugs of abuse

Abbreviations: aps – average particle size, I.D. – Internal diameter, C18 – octadecyl, HSS – High strength silica, PFP – pentafluorophenyl, BEH – Ethylene Bridged Hybrid, EC – End capped

No. of analytes	LC Column Details	Reference
30	C18 (50 x 3.0 I.D. mm; 2.5 µm aps, Waters Xterra)	Feng *et al.* (2007)
35	HSS T3 (100 x 2.1 I.D. mm, 1.8 µm aps, Waters Acquity)	Hegstad *et al.* (2014)
38	Phenyl-hexyl (100 x 2.1 I.D. mm, 2.6 µm aps, ThermoFisher Accucore)	Helfer *et al.* (2016)
38	C18 (50 x 2.1 I.D. mm, 1.6 µm aps, Cortex)	Jagerdeo and Schaff (2016)
65	PFP (50 x 2.1 I.D. mm, 3 µm aps, ThermoFisher Hypersil Gold)	Li *et al.* (2013)
67	C18 (100 x 3 I.D. mm, 1.8 µm aps, Zorbax Eclipse Plus)	Marin *et al.* (2012)
37	C18 (100 x 2.1 I.D. mm, 2.7 µm aps, Agilent Technologies Poroshell)	McMillin *et al.* (2015)
356	Phenyl-hexyl (100 x 3 I.D. mm, 3 µm aps, ThermoFisher Betasil)	Mueller *et al.* (2011)
616	Phenyl-hexyl (100 x 3 I.D. mm, 3 µm aps, ThermoFisher Betasil)	Roche *et al.* (2016)
33	BEH Phenyl (50 x 2.1 I.D. mm, 1.7 µm aps, Waters Acquity)	Rosano *et al.* (2016)
27	C18 (50 x 2.1 I.D. mm, 5 µm aps, ThermoFisher Hypersil Gold)	Schaefer *et al.* (2013)
35	EC-C18 (50 x 3.0 I.D. mm, 2.7 µm aps, Agilent Technologies Poroshell)	Shin *et al.* (2014)
21	BEH C18 (50 x 2.1 I.D. mm, 1.7 µm aps, Waters Acquity)	Valen *et al.* (2016)

Three columns were selected for evaluation during initial method development; C18, PFP and phenyl-hexyl (all 100 x 2.1 I.D. mm, ThermoFisher). To compare the columns, a simple gradient elution profile (2-100 % organic eluent [0.1 % (v/v) formic acid in acetonitrile:methanol (1+1)] over 15 minutes) was used, with 10 mmol/L ammonium formate with 0.1 % (v/v) formic acid as the aqueous eluent. A portion (50 µL) of an aqueous solution containing all analytes (100 µg/L) was analysed. Methadone and EDDP were strongly retained on the PFP column leading to significant carryover for these analytes on subsequent analyses. In addition, retention of morphine glucuronide was poorer on the PFP column (Figure 2.3). For most analytes the phenyl-hexyl column provided sharper chromatographic peaks than the C18 column, and thus was selected for further method development.

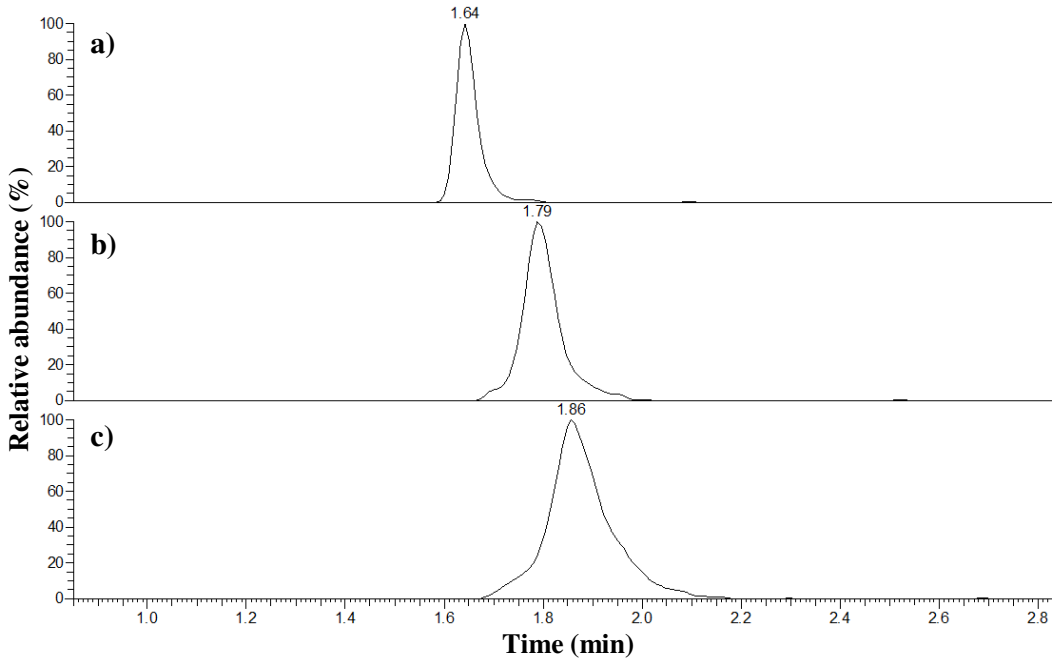
Figure 2.3 – Extracted ion chromatograms to show the retention of morphine glucuronide (*m/z* 462.1759) on a) C18, b) phenyl-hexyl, and c) PFP columns [all 100 x 2.1 I.D. mm, ThermoFisher]



2.3.2.2 Eluent Selection

When first testing the phenyl-hexyl column, a simple gradient (2-100 % organic eluent [0.1 % (v/v) formic acid in acetonitrile:methanol (1+1)] over 15 minutes) was used. The column temperature was maintained at 40 ^{o}C, and the total LC flow rate was 0.3 mL/min. 10 mmol/L ammonium formate with 0.1 % (v/v) formic acid was used as the aqueous eluent. Three organic eluents were investigated; 0.1 % (v/v) formic acid in (i) acetonitrile, (ii) acetonitrile:methanol (1+1), and (iii) methanol. Chromatographic peaks broadened with increasing methanol content, particularly the early eluting peaks (e.g. morphine, Figure 2.4). However, retention of morphine glucuronide was poor when using solely acetonitrile as the organic eluent. As a compromise 0.1 % (v/v) formic acid in acetonitrile:methanol (1+1) was selected for the organic eluent.

Figure 2.4 – Extracted ion chromatograms for morphine (*m/z* 286.1438) using 0.1 % (v/v) formic acid in a) acetonitrile, b) acetonitrile:methanol (1+1), and c) methanol as the organic eluent [Phenyl-hexyl (100 x 2.1 I.D. mm) column, ThermoFisher]



2.3.3 Mass Spectrometry Method Development

2.3.3.1 Ionisation Mode and Polarity Switching

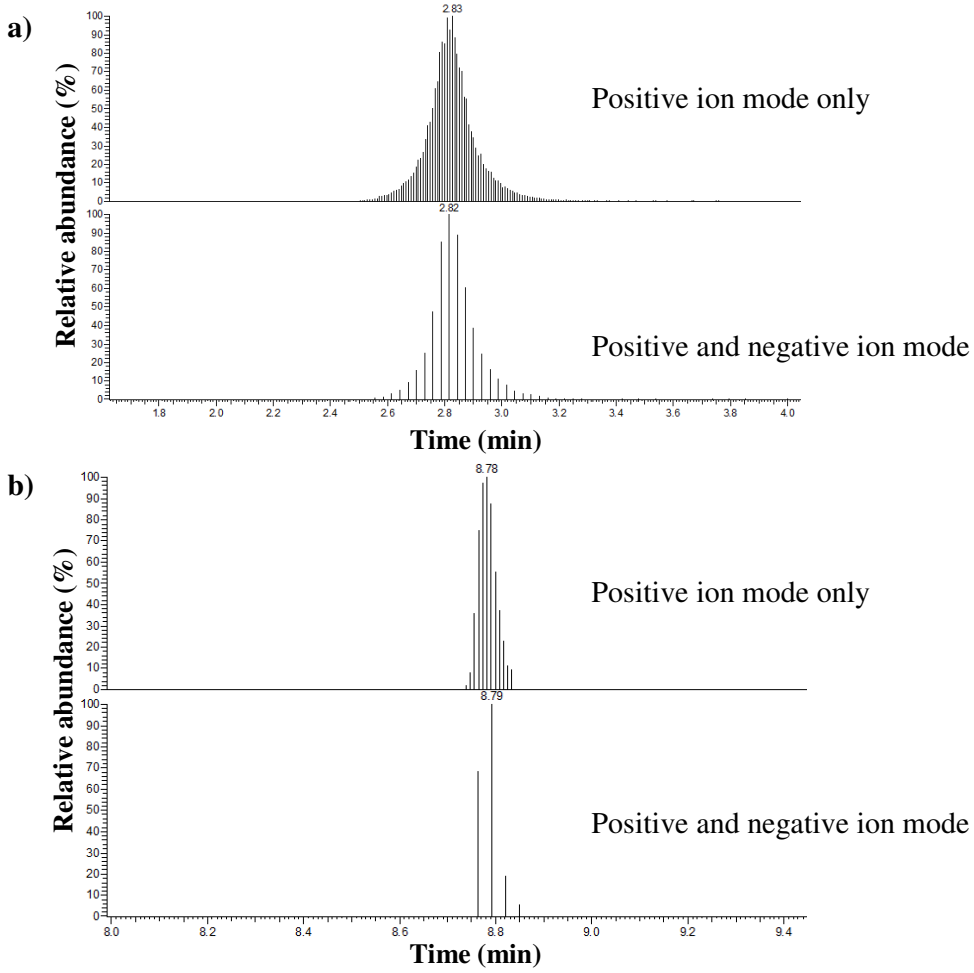
Most of the analytes studied are basic and ionise in positive mode. Barbiturates, however, are weakly acidic and will only ionise in negative mode. In order to detect all analytes, the method would need to include both positive and negative mode scans. However, the time taken to switch between polarities resulted in a severe reduction in the data points that could be obtained over the chromatographic peaks. This was particularly apparent for low concentration analytes such as buprenorphine (Figure 2.5).

Instead of including negative ion scans over the whole analysis, the possibility of including negative scan segments at only the relevant times when barbiturates elute was explored. The retention times of the barbiturates ranged from 8.0-9.5 min, during which many other analytes eluted (e.g. buprenorphine, EDDP), thus splitting positive and negative ion scans into different time segments was not feasible in practice.

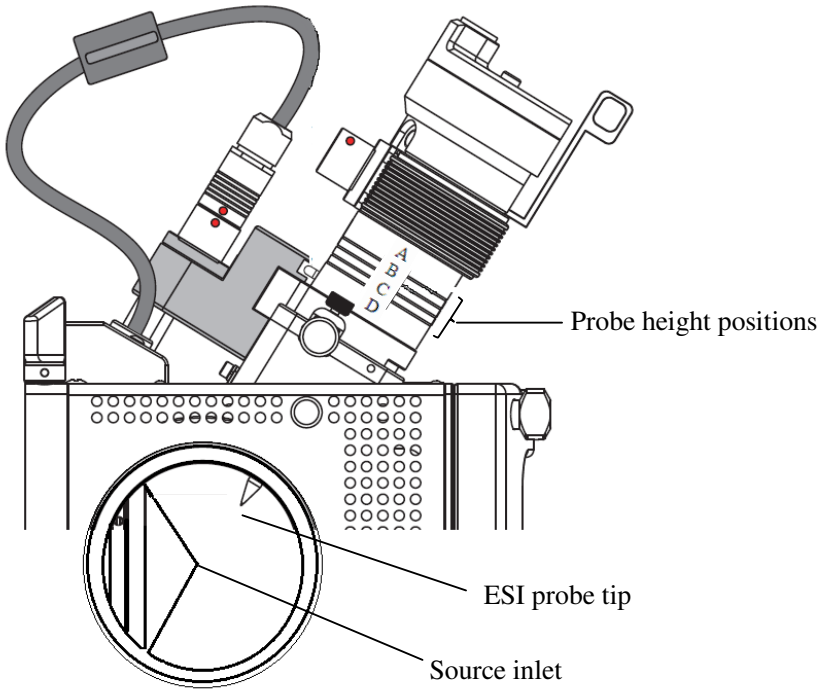
2.3.3.2 Optimisation of Source Conditions

The majority of source parameters are optimised according to the LC flow rate. The parameters that are more analyte-specific include the ion transfer capillary temperature and the positioning of the ESI probe. The ion transfer capillary temperature is critical for the majority of compounds. When the temperature is too low, ions may not be fully desolvated. Higher temperatures may improve desolvation for some analytes but may also start to degrade more thermally labile analytes. The distance between the ESI probe and the source inlet may be set at 4 different positions (A, B, C, or D, Figure 2.6), with the distance typically greatest when a higher LC flow rate is used (position D).

<u>Figure 2.5</u> – Analysis of the cutoff solution to compare the number of data points over a) morphine, and b) buprenorphine chromatographic peaks when collecting data in positive and negative ion mode compared to only positive ion mode



<u>Figure 2.6</u> – Schematic illustration of the ESI probe and source housing on the Q-Exactive mass spectrometer



The capillary temperature was investigated at 250 and at 350 ^{o}C. Analyte peak area in the low and cutoff calibrator solutions was compared at the two temperatures to ascertain the optimum value to use (Figure 2.7). The amfetamine and metamfetamine peak areas were significantly reduced when the capillary was at 350 ^{o}C. As a result, the capillary temperature was set at 250 ^{o}C for the method.

To ascertain the optimum distance of the ESI probe from the mass spectrometer source, the cutoff calibrator solution was analysed with the probe at three different positions (B, C and D; D being the furthest distance from the source). Most analytes showed increased peak area when the probe was further from the source, thus position D was selected for the method (Figure 2.8).

To ascertain the other source conditions, including gas flow rates, spray voltage and vaporiser temperature, the instrument software was used to give the default settings based on a LC flow rate of 0.3 mL/min.

Figure 2.7 – The influence of capillary temperature on analyte peak area in the low calibrator (light grey) and cutoff (dark grey) solutions

The graph compares peak areas with the capillary temperature at 350 °C as opposed to 250 °C

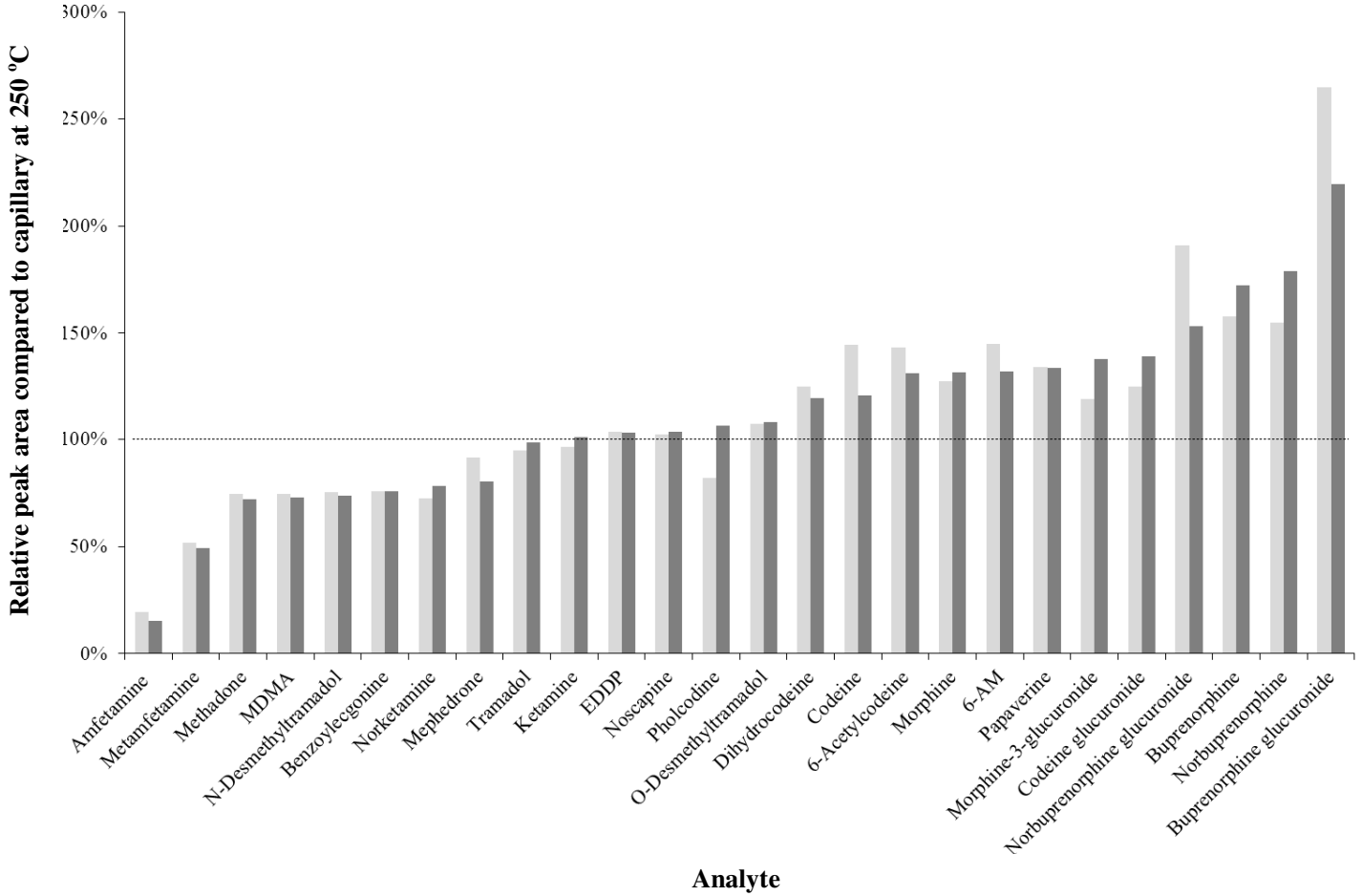
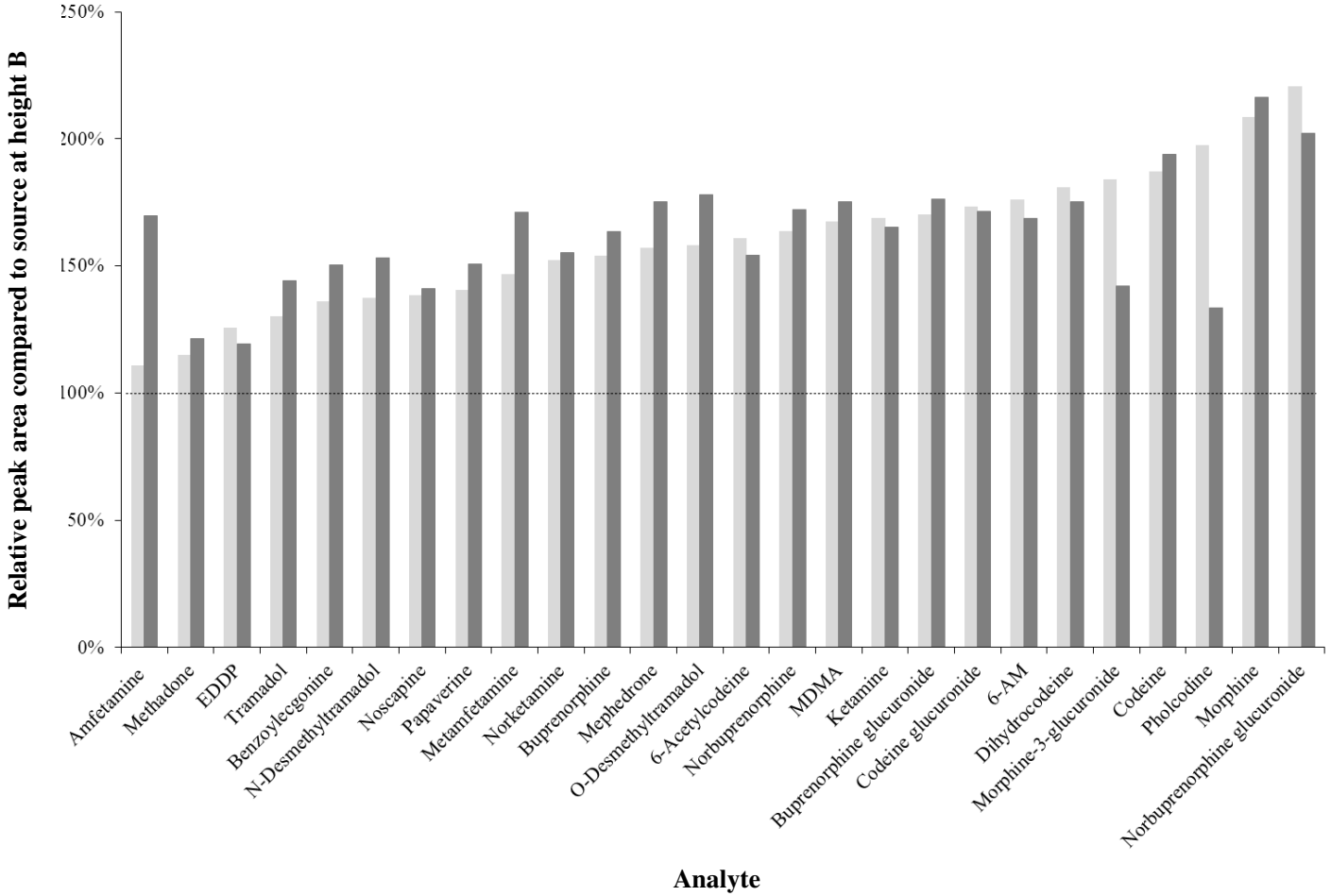


Figure 2.8 – The influence of increasing the distance of the ESI probe from the source on analyte peak area in the cutoff solution

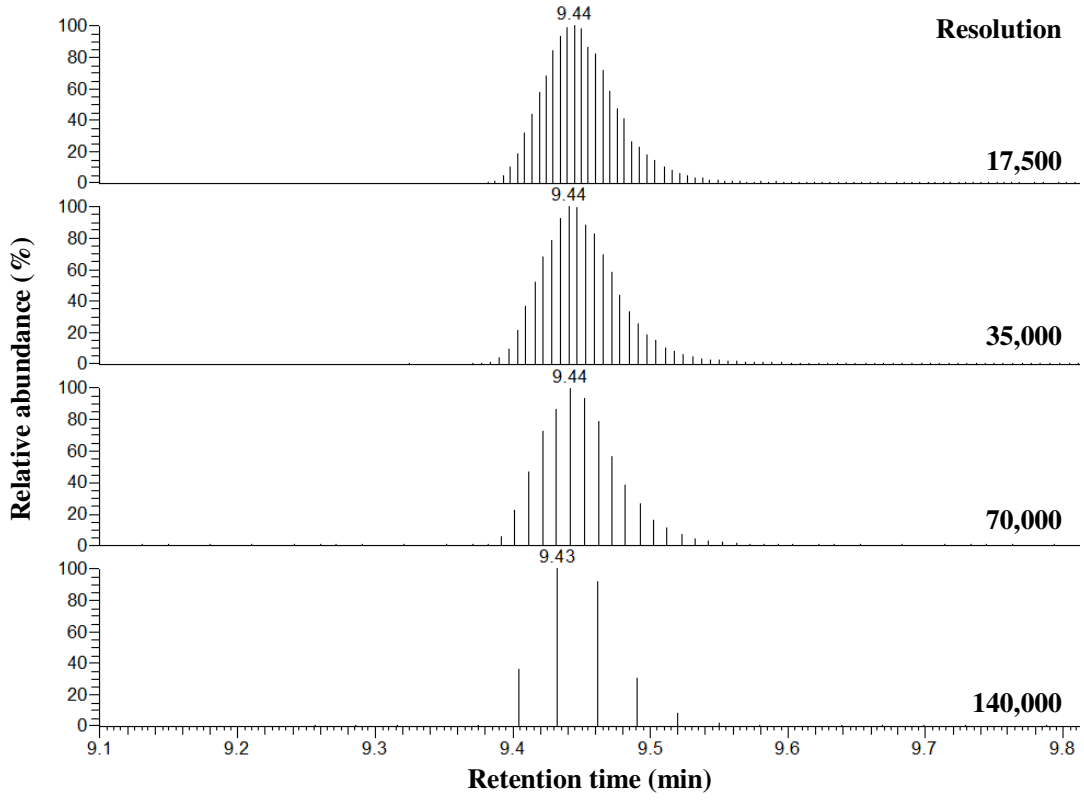
Three positions were investigated; B, C, and D. The graph compares peak areas with the probe at position C (pale grey) and D (dark grey) against B



2.3.3.3 Mass Resolution

Mass resolution relates to the ability of a mass spectrometer to separate peaks of one mass from another, with higher resolution giving improved selectivity. However, increasing mass resolution increases the time required for each scan. To identify unknown substances, the mass resolution needs to be as high as possible to give the greatest selectivity. However, there must still be enough data points across chromatographic peaks to enable accurate quantitation. To ascertain the optimum resolution setting for the full scan experiment, the cutoff calibrator was analysed at each resolution setting and the number of data points under chromatographic peaks visually compared (Figure 2.9)

Figure 2.9 – Extracted ion chromatograms (*m/z* 310.2165) to show the reduction in MS scans collected over the methadone peak due to an increase in mass resolution (theoretical resolution stated, defined as FWHM at *m/z* 200)



2.3.3.4 Product Ions

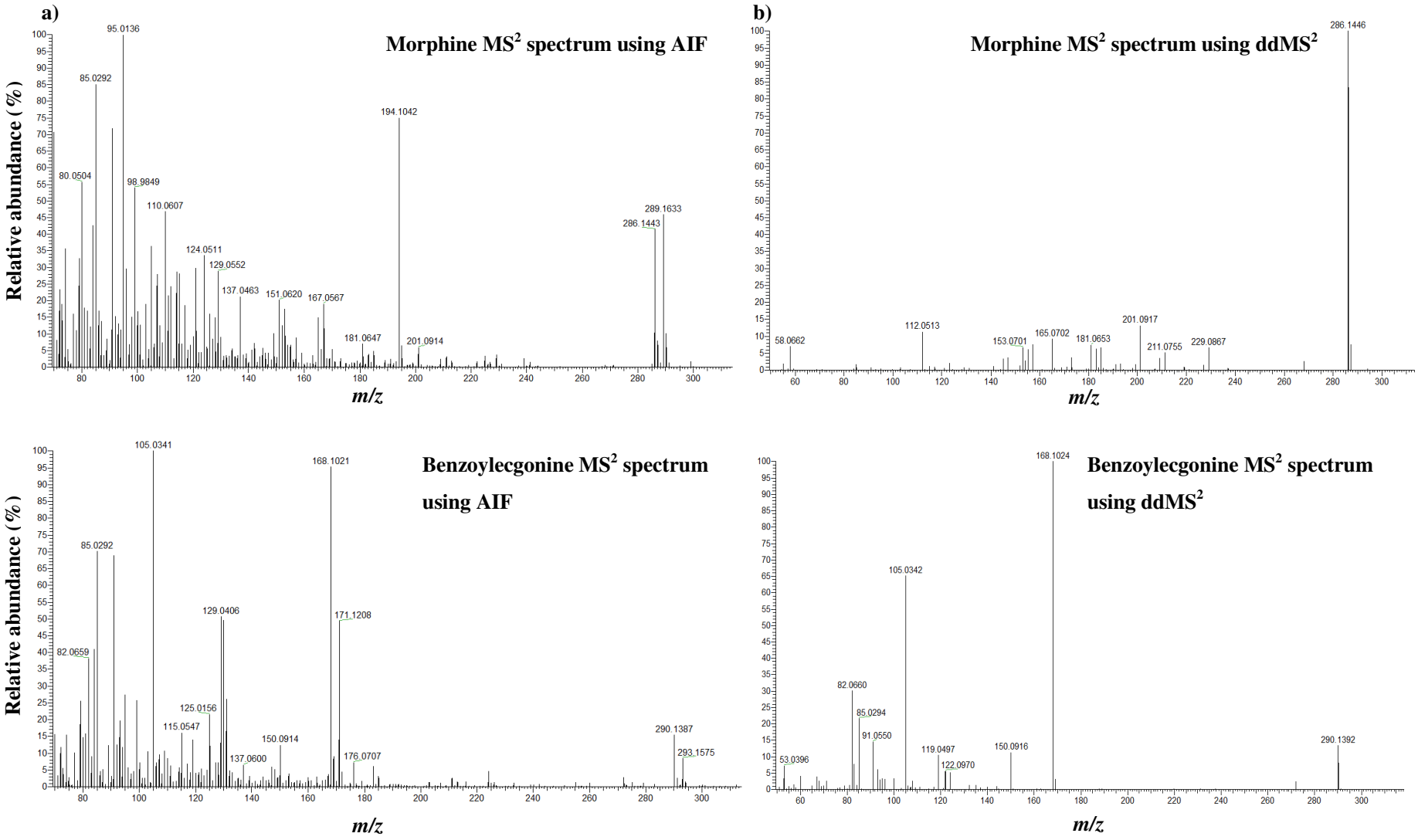
Product ions may be used to confirm peak identity. Two types of fragmentation scan experiments were evaluated to decide that to use in the method; data-dependent MS^2 ($ddMS^2$) and all-ion fragmentation (AIF). $ddMS^2$ scan experiments fragment the top 5 precursor ions (based on intensity) in the preceding full scan, whereas AIF experiments fragment all the precursor ions seen in the preceding full scan.

For both experiments, conditions in the HCD cell were set as follows; collision gas: nitrogen, stepped normalized collision energy (NCE): 35 V [±50 %], and settings for the MS^2 data collection were: resolution 17,500, maximum injection time 200 ms, AGC target 3 x 10^6 ions. The scan range for the AIF experiment was set as 70-750 *m/z*.

The $ddMS^2$ scans had less background noise, particularly at low *m/z* values, and as a result the MS^2 spectra were clearer (Figure 2.10). However, if an analyte of interest is at low concentration or co-elutes with other analytes, an MS^2 scan may not be triggered when using $ddMS^2$. Additionally, when using $ddMS^2$ the number of full scan spectra will be reduced as 5 separate MS^2 scans are conducted after each full scan, as opposed to 1 MS^2 scan when using AIF. AIF has the further advantage of detecting all product ions present, but as the fragmentation is not specific (i.e. resultant product ions in the MS^2 spectra are from all compounds eluting at that time) it is difficult to associate product ions to specific precursor ions.

To capture as much information as possible, AIF was used in the developed method. An important consideration when using AIF was to ensure that confirming product ions for analytes were not generated by the corresponding internal standard to minimise the possibility of false positive results. Individual aqueous solutions of analytes and internal standards (all 100 µg/L) were analysed using $ddMS^2$ to identify specific product ions.

Figure 2.10 – Comparison of the MS^2 spectra produced for morphine and benzoylecgonine when using a) AIF, and b) $ddMS^2$ scan settings



2.3.4 Selection of 'cutoff' concentrations

Many guidelines for recommended drug cutoff concentrations have been published. The concentration used is largely dictated by whether the sample is submitted for clinical reasons or for workplace drug testing, with the latter usually having lower cutoff concentrations. The suggested cutoff concentrations also differ according to the methodology being used, i.e. whether the test is for screening (largely based on immunoassay) or confirmation (largely based on chromatographic assays) purposes. A summary of recommended cutoff concentrations is given in Table 2.5. Where no formal cutoff concentrations were documented, a literature search was conducted to establish urine concentrations and provide an evidence-based cutoff concentration for analytes.

No clear guidance exists for urinary cutoff concentrations for street heroin markers (e.g. 6-acetylcodeine, noscapine, and papaverine). Urinary 6-acetylcodeine concentrations are typically lower than 6-AM concentrations. One method stated a lower limit of quantification (LLoQ) of 1 µg/L for 6-acetylcodeine and most samples tested had 6-acetylcodeine concentrations less than 30 µg/L (O'Neal and Poklis, 1997; 1998). Noscapine and papaverine are also present at low concentration in urine from heroin users because they undergo extensive metabolism with less than 1 % of a dose being excreted unchanged in urine (Belpaire *et al.*, 1978; Tsunoda and Yoshimura, 1981). The cutoff for street heroin metabolites was assigned as 10 µg/L, to be concordant with the cutoff for 6-AM. The full list of cutoff concentrations for the method developed is given in Table 2.6.

Table 2.5 – Recommended analyte 'cutoff' concentrations for drug screening and confirmation in urine samples

[a]Dependent on specific opioid, total morphine 300 µg/L, total codeine or dihydrocodeine 2000 µg/L

[‡]Presence of any metabolite above cutoff (i.e. norbuprenorphine, buprenorphine glucuronide, and/or norbuprenorphine glucuronide)

Analyte/ Analyte Group	Workplace/ Clinical setting	Cutoff concentration (µg/L)		Source
		Screening	Confirmation	
6-AM	Workplace	-	10	EWDTS (2002)
	Workplace	-	10	EWDTS (2015)
	Clinical	-	10	LGC Standards (2016)
	Workplace	10	10	SAMHSA (2010)
Amfetamines	Workplace	500	200	EWDTS (2002)
	Workplace	500	200	EWDTS (2015)
	Clinical	1000	1000	LGC Standards (2016)
	Workplace	500	250	SAMHSA (2010)
Barbiturates	Workplace	200	150	EWDTS (2002)
	Workplace	200	250	EWDTS (2015)
	Clinical	300	300	LGC Standards (2016)
Benzodiazepines	Workplace	200	100	EWDTS (2002)
	Workplace	200	100	EWDTS (2015)
	Clinical	300	300	LGC Standards (2016)
Buprenorphine/ buprenorphine metabolites[‡]	Workplace	5/5	5/5	EWDTS (2002)
	Workplace	5/5	2/2	EWDTS (2015)
	Clinical	5/5	5/5	LGC Standards (2016)
Cannabis	Workplace	50	15	EWDTS (2002)
	Workplace	50	15	EWDTS (2015)
	Clinical	50	15	LGC Standards (2016)
	Workplace	50	15	SAMHSA (2010)
Cocaine	Workplace	300	150	EWDTS (2002)
	Workplace	150	150	EWDTS (2015)
	Clinical	300	300	LGC Standards (2016)
	Workplace	150	100	SAMHSA (2010)
Ketamine	Workplace	-	-	EWDTS (2002)
	Workplace	-	-	EWDTS (2015)
	Clinical	-	-	LGC Standards (2016)
	Clinical	50	100	Lin *et al.* (2010)
Methadone/ EDDP	Workplace	300/300	250/250	EWDTS (2002)
	Workplace	300/100	250/75	EWDTS (2015)
	Clinical	300/300	300/300	LGC Standards (2016)
Opioids	Workplace	300	300	EWDTS (2002)
	Workplace	300	300	EWDTS (2015)
	Clinical	300	300/2000[a]	LGC Standards (2016)
	Workplace	2000	2000	SAMHSA (2010)
Tramadol	Workplace	-	-	EWDTS (2002)
	Workplace	-	-	EWDTS (2015)
	Clinical	-	-	LGC Standards (2016)
	Clinical	100	50	Beck *et al.* (2014)

Table 2.6 – Drug screening assay: Cutoff concentrations selected for the LC-HRMS method developed

* Dihydrocodeine glucuronide concentration estimated based upon dihydrocodeine calibration curve
# Conversion to buprenorphine equivalents and summed
† Norketamine converted to ketamine equivalent and summed
‡ Metabolites converted to tramadol equivalents and summed

Analyte	Cutoff (µg/L)
Amfetamine	200
Metamfetamine	200
MDMA	200
Mephedrone	200
Total morphine	300
Total codeine	300
Total dihydrocodeine*	300
6-AM	10
Pholcodine	300
Methadone	250
EDDP	250
Benzoylecgonine	150
Buprenorphine	5
Buprenorphine metabolites#	5
Ketamine & norketamine†	50
Tramadol & metabolites‡	200
Street heroin	10

2.3.5 Selection of Internal Standards

Stable isotope-labelled analogues of some analytes were selected as internal standards (Table 2.7). Naturally-occurring isotopes of an analyte (e.g. those containing ^{13}C instead of ^{12}C) may result in interference with the internal standard (Duxbury *et al.*, 2008). To minimise this risk, the compounds chosen were at least 3 amu different from the corresponding analyte.

Table 2.7 – Drug screening assay: Internal standards and associated analytes

Internal standard	Analyte/s
Amfetamine-$^{13}C_6$	Amfetamine
Metamfetamine-D_{14}	Metamfetamine
MDMA-D_5	MDMA
Mephedrone-D_3	Mephedrone
Morphine-D_3	Morphine, morphine-3-glucuronide, pholcodine
Codeine-D_6	Codeine, codeine glucuronide
Dihydrocodeine-D_6	Dihydrocodeine
6-AM-D_3	6-AM, 6-acetylcodeine, noscapine, papaverine
Methadone-D_3	Methadone, EDDP
Benzoylecgonine-D_3	Benzoylecgonine
Buprenorphine-D_4	Buprenorphine, buprenorphine glucuronide
Norbuprenorphine-D_3	Norbuprenorphine, norbuprenorphine glucuronide
Ketamine- D_4	Ketamine
Norketamine-$^{13}C_6$	Norketamine
Tramadol-^{13}C-D_3	Tramadol, *O*-desmethyltramadol, *N*-desmethyltramadol

For amfetamine initially two deuterated analogues, amfetamine-D_5 and amfetamine-D_{11}, were investigated for use as an internal standard. Neither analogue co-eluted with amfetamine (Figure 2.11). As a result, matrix effects were not fully compensated for using either internal standard (Table 2.8). To overcome this issue, a ^{13}C-labelled analogue, amfetamine-$^{13}C_6$, was purchased. Amfetamine-$^{13}C_6$ co-eluted with amfetamine and as a result matrix effects were better compensated for. The relative peak area of amfetamine to that of each stable isotope was assessed in 10 urine samples (all analytes 200 µg/L) and showed large variation with both deuterated analogues, which could cause inaccurate results (Table 2.9). In contrast, the relative peak area of amfetamine to amfetamine-$^{13}C_6$ was consistent in all 10 samples, with a RSD of 2 %.

Figure 2.11 – Chromatographic separation of amfetamine (black, 5.8 min) and different stable isotopes; amfetamine-$^{13}C_6$ (red, 5.8 min), amfetamine-D_5 (green, 5.7 min) and amfetamine-D_{11} (blue, 5.6 min)

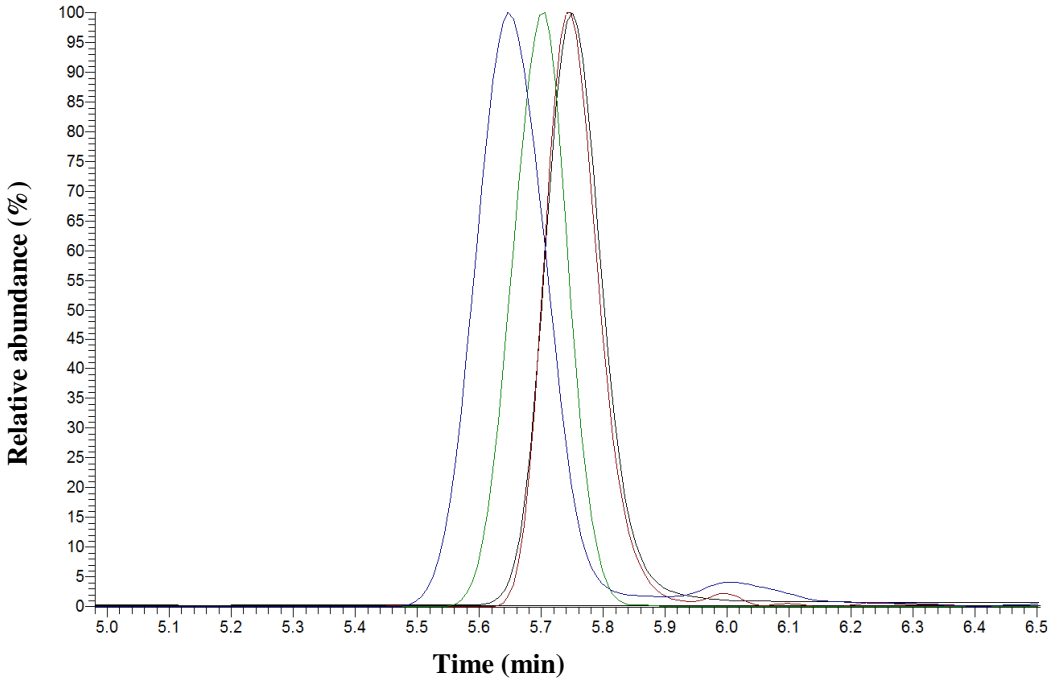


Table 2.8 – Observed matrix effects for amfetamine and selected stable isotopes

Analyte	Mean Matrix effect (%)	Relative matrix effect to Amfetamine (%)
Amfetamine	78	-
Amfetamine-$^{13}C_6$	79	101
Amfetamine-D_5	87	112
Amfetamine-D_{11}	88	113

Table 2.9 – Variation in the relative peak area of amfetamine to different stable isotopes between 10 urine samples containing amfetamine, amfetamine-$^{13}C_6$, amfetamine-D_5, amfetamine-D_{11} (all 200 µg/L)

	Relative peak area of amfetamine to internal standard		
	Amfetamine-$^{13}C_6$	Amfetamine-D_5	Amfetamine-D_{11}
1	0.84	0.79	0.68
2	0.79	0.86	0.98
3	0.84	0.79	0.65
4	0.85	0.73	0.60
5	0.85	0.78	0.65
6	0.83	0.70	0.59
7	0.84	0.77	0.68
8	0.84	0.93	0.86
9	0.83	0.73	0.63
10	0.84	1.02	0.98
Mean	0.83	0.81	0.73
SD	0.02	0.10	0.15
% RSD	2	12	21

2.4 Sample Preparation

A literature search was performed to ascertain the sample preparation methods that had been used for urine drugs of abuse screening. Most methods used either solid-phase extraction, or sample dilution. As one of the aims of the method was to enable retrospective detection of analytes (i.e. retrospective data interrogation), it was decided to use a non-selective sample preparation method to ensure that unknown substances were not missed as a result of using solid-phase extraction.

Published methods for drug analysis in urine have used 5-fold (Andersson *et al.*, 2008; Gustavsson *et al.*, 2007; Svensson *et al.*, 2007), 10-fold (Eichhorst *et al.*, 2009), or 50-fold (Politi *et al.*, 2007) dilution. 50-fold dilution was considered too great as some analytes (e.g. buprenorphine) are present at low concentration during abstinence treatment. Thus, a 10-fold dilution was adopted for use in the method developed to minimise matrix effects as far as possible without compromising assay sensitivity.

2.5 Practical Considerations

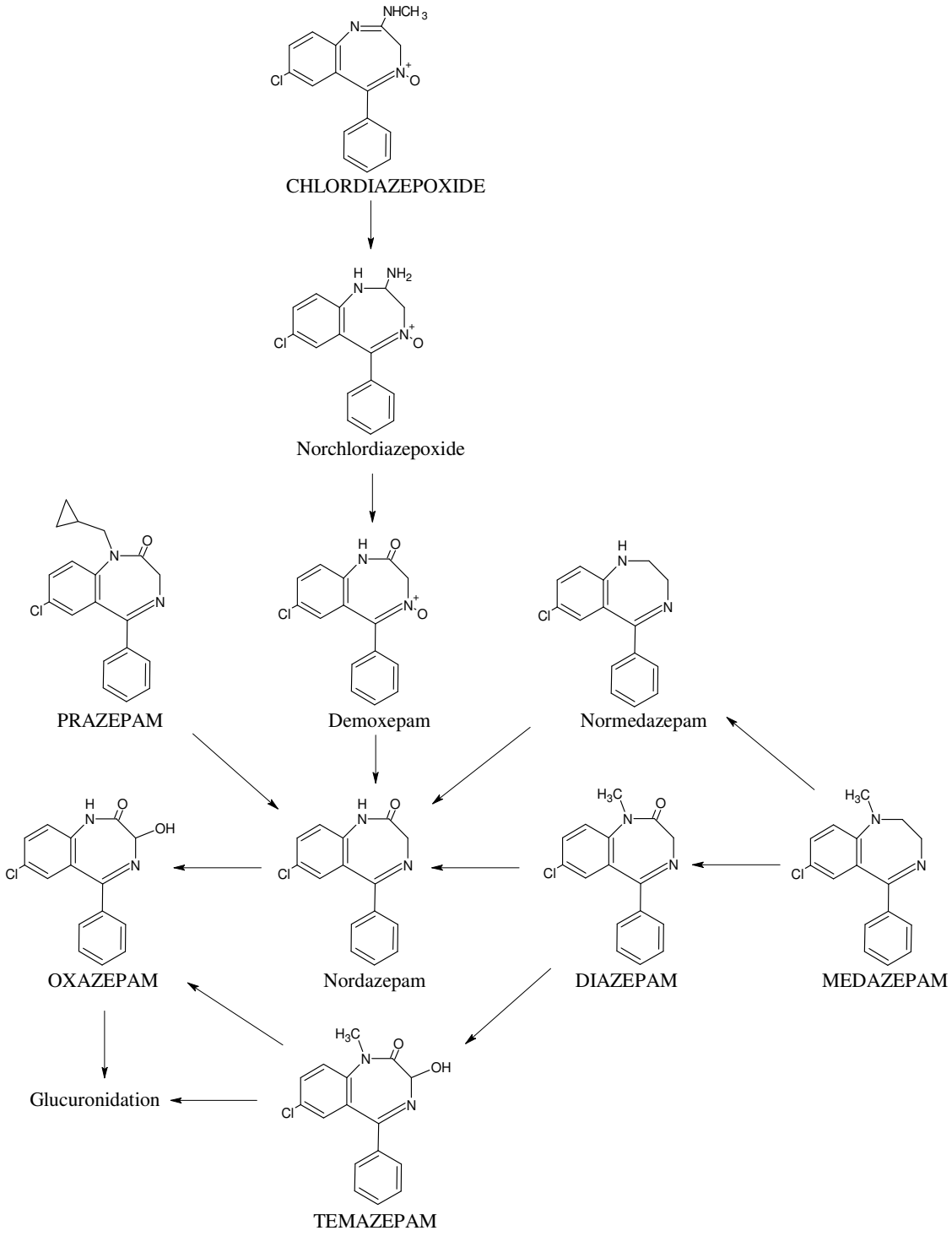
2.5.1 Benzodiazepines

Development of a comprehensive screening method for benzodiazepines is complicated due to the large number of benzodiazepine drugs prescribed and used. In addition, benzodiazepines undergo extensive metabolism and as a result many metabolites are found in urine. Interpretation of results is also challenging as identifying the drug ingested can be difficult since many benzodiazepines share a common metabolic pathway (Figure 2.12). For example, detection of oxazepam glucuronide is possible following ingestion of diazepam, chlordiazepoxide and/or temazepam as well as following ingestion of oxazepam itself. Detection of unique metabolites, e.g. demoxepam from chlordiazepoxide, or in some cases the parent drug itself can aid the interpretation of analytical results.

Whilst detection of benzodiazepine drugs is possible with the method developed, it was thought that more than 20 benzodiazepine analytes would have to be included in the assay to provide comparable results to the immunoassay currently in use. The time taken for checking peak integrations, and then for interpretation of results was considered too great for a routine service. For most cases, identification of an individual benzodiazepine that has been ingested is not clinically significant. Specific identification may be useful when an individual is prescribed a benzodiazepine, but in addition misuse of a different benzodiazepine is suspected. The best approach may be to offer an additional test for benzodiazepine differentiation that can be requested when there is a clinical need.

Figure 2.12 – Common metabolic pathway of selected benzodiazepine drugs

Drugs that may be prescribed in the UK are shown capitalised.

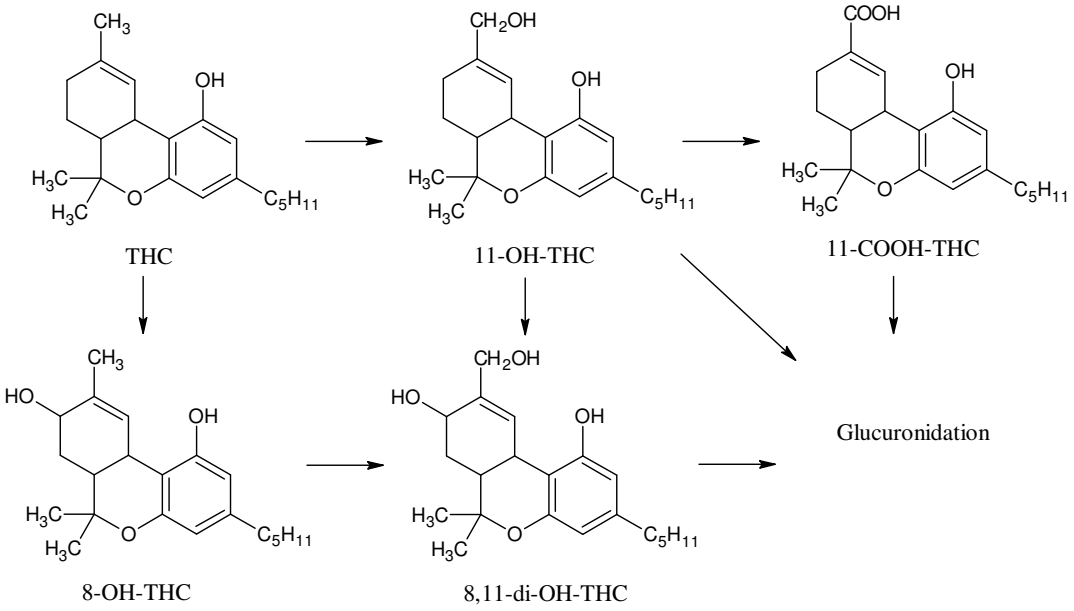


2.5.2 Cannabis

Tetrahydrocannabinol (THC), the main psychoactive constituent of cannabis, is rapidly and extensively metabolised after administration (Figure 2.13). The major urinary metabolite is 11-COOH-THC glucuronide, with assays typically targeting this analyte or 11-COOH-THC itself after urine hydrolysis.

<u>Figure 2.13</u> – Metabolic pathway of tetrahydrocannabinol in man

Key: tetrahydrocannabinol (THC), 11-hydroxy tetrahydrocannabinol (11-OH-THC), 11-nor-9-carboxy tetrahydrocannabinol (11-COOH-THC), 8-hydroxy tetrahydrocannabinol (8-OH-THC), 8,11-dihydroxy tetrahydrocannabinol (8,11-di-OH-THC)



11-OH-THC, 11-COOH-THC and 11-COOH-THC glucuronide showed good chromatographic retention using the method developed (Figure 2.14). It was noted that in-source breakdown of the glucuronide to parent drug occurred, but this was not deemed problematic as 11-COOH-THC and the glucuronide were resolved chromatographically. However, when analysing these compounds at relevant concentrations in urine samples the sensitivity of the assay was poor (Figure 2.15). The current cutoff concentration in use with the immunoassay method is 50 µg/L, with the developed LC-HRMS method only 11-COOH-THC had a true chromatographic peak distinguishable from background noise at 50 µg/L.

Figure 2.14 – Extracted ion chromatograms of individual aqueous solutions (each 1 mg/L) of a) 11-OH-THC, b) 11-COOH-THC, and c) 11-COOH-THC glucuronide

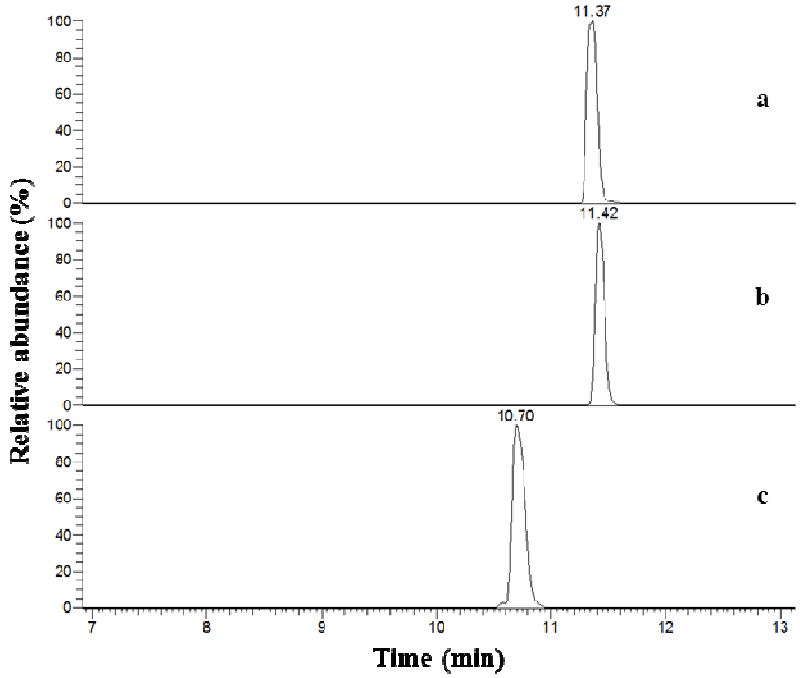
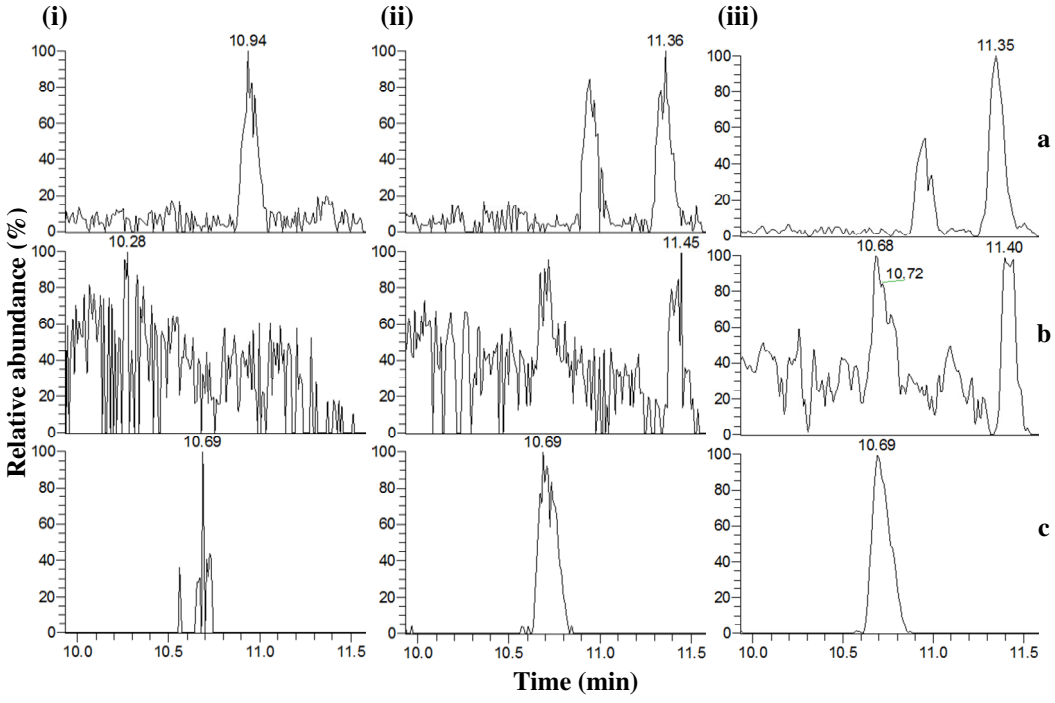


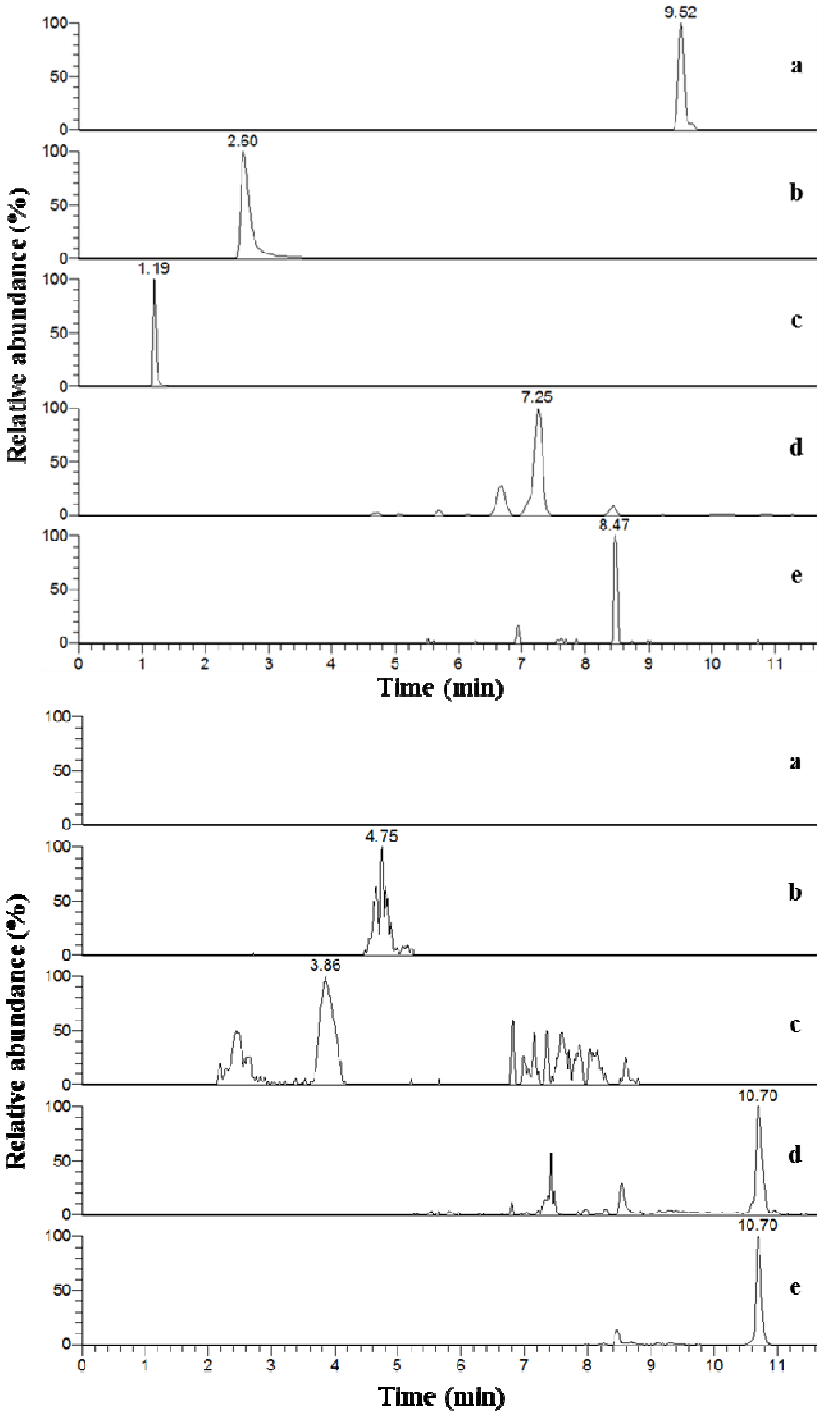
Figure 2.15 – Extracted ion chromatograms to show a) 11-OH-THC, b) 11-COOH-THC, and c) 11-COOH-THC glucuronide in human urine at (i) 25 µg/L, (ii) 50 µg/L, and (iii) 100 µg/L



Time constraints associated with implementation of the developed LC-HRMS method into routine service meant that further work to try and improve sensitivity could not be conducted. Due to this, the immunoassay remained in use for cannabis detection.

The immunoassay exhibits good sensitivity, but false positives may arise due to the presence of the antiretroviral drug efavirenz in urine samples (Oosthuizen and Laurens, 2012; Rossi *et al.*, 2006). Efavirenz (Sustiva®, Bristol-Myers Squibb) is prescribed on its own or as a combination tablet (Atripla®, Bristol-Myers Squibb) also containing emtricitabine and tenofovir. Retrospective interrogation of LC-HRMS data can be undertaken to confirm the presence of these drugs if a positive immunoassay cannabinoid result is queried (Figure 2.16).

<u>Figure 2.16</u> – Extracted ion chromatograms to show the presence of efavirenz (a), emtricitabine (b), and tenofovir (c) in the absence of THC-COOH (d) and its glucuronide (e) in a patient urine sample (top) which screened positive for cannabis by CEDIA (apparent cannabinoid concentration = 76 µg/L), compared to a patient urine sample (bottom) which was confirmed positive by the presence of THC-COOH glucuronide (apparent cannabinoid concentration = 92 µg/L)



2.6 Method Validation

Intra- and inter-assay accuracy and precision are summarised in Tables 2.10 and 2.11. The accuracy for analytes present in past EQA samples ranged from 79-118 % (Table 2.12). The LoD for all analytes ranged between 2.5-63 µg/L. The LoD of individual analytes are summarised in Table 2.13, which also details the cutoff concentrations selected for the method (Section 2.3.4). For all analytes the LoD was at least half the concentration of the cutoff concentration. Typical chromatograms for all analytes are shown in Figure 2.17. Typical mass accuracies are given in Table 2.14. For most analytes, carryover was less than 20 %. However, significant carryover was seen for buprenorphine (128 %) and norbuprenorphine (39 %). When buprenorphine is taken therapeutically, urine buprenorphine concentration is low, often less than 5 µg/L. However, in cases of sample adulteration with buprenorphine, urine buprenorphine concentration may be 50 mg/L or more. In these cases, subsequent samples have to be repeated to ensure that the possibility of carry-over has been eliminated. The internal standards used were seen to compensate well for ion suppression/enhancement for all analytes (Table 2.15).

Table 2.10 – Drug screening assay: Intra-assay accuracy and precision data

Accuracy (% nominal QC values) and precision (% RSD)

Analyte	Nominal (µg/L)	Mean measured (µg/L)	Accuracy (%)	RSD (%)
Amfetamine	150	145	97	5
	200	214	107	8
	250	268	107	4
Metamfetamine	150	154	103	4
	200	225	113	8
	250	281	112	3
MDMA	150	146	98	3
	200	205	103	7
	250	251	101	2
Mephedrone	150	136	91	3
	200	198	99	5
	250	225	90	2
Morphine	225	231	102	2
	300	291	97	7
	375	388	104	1
Morphine-3-glucuronide	225	213	95	4
	300	293	98	7
	375	353	94	11
Codeine	225	235	105	2
	300	290	97	6
	375	396	106	2
Codeine glucuronide	225	232	103	3
	300	296	99	6
	375	372	99	2
Dihydrocodeine	225	239	106	3
	300	301	100	7
	375	375	100	2
6-AM	7.5	8	109	2
	10	10	97	7
	12.5	14	110	2
Pholcodine	225	221	98	4
	300	299	100	7
	375	347	93	6
Methadone	188	173	92	2
	250	240	96	4
	313	281	90	4
EDDP	188	175	93	2
	250	246	99	5
	313	266	85	3
Benzoylecgonine	113	119	106	2
	150	155	103	6
	188	204	109	2

Table 2.10 (cont.) – Drug screening assay: Intra-assay accuracy and precision data

Accuracy (% nominal QC values) and precision (% RSD)

Analyte	Nominal (µg/L)	Mean measured (µg/L)	Accuracy (%)	RSD (%)
Buprenorphine	3.25	4.2	113	6
	5.00	4.6	93	5
	6.75	6.0	97	6
Norbuprenorphine	3.25	4.5	119	3
	5.00	4.7	93	7
	6.75	6.4	102	3
Buprenorphine glucuronide	3.25	4.5	119	4
	5.00	4.9	97	8
	6.75	6.4	102	4
Norbuprenorphine glucuronide	3.25	4.2	112	7
	5.00	5.2	104	9
	6.75	6.1	98	8
Ketamine	38	39	105	3
	50	51	102	7
	63	58	93	3
Norketamine	38	37	99	3
	50	50	101	6
	63	56	90	2
Tramadol	150	141	94	3
	200	203	102	6
	250	227	91	3
O-Desmethyltramadol	150	164	109	2
	200	199	99	6
	250	253	101	4
N-Desmethyltramadol	150	162	108	3
	200	207	104	7
	250	251	100	3
6-Acetylcodeine	7.5	8	105	4
	10	10	96	8
	12.5	13	107	3
Noscapine	7.5	7	99	3
	10	9	95	6
	12.5	12	97	3
Papaverine	7.5	7	96	3
	10	10	97	7
	12.5	12	96	2

Table 2.11 – Drug screening assay: Inter-assay accuracy and precision data

Accuracy (% nominal QC values) and precision (% RSD)

Analyte	Nominal (µg/L)	Mean measured (µg/L)	Accuracy (%)	RSD (%)
Amfetamine	150	160	107	1
	200	218	109	7
	250	294	118	10
Metamfetamine	150	145	96	5
	200	203	101	8
	250	262	105	8
MDMA	150	150	100	7
	200	197	98	5
	250	257	103	12
Mephedrone	150	150	100	10
	200	190	95	9
	250	249	100	12
Morphine	225	241	107	7
	300	288	96	3
	375	408	109	10
Morphine-3-glucuronide	225	232	97	13
	300	282	94	6
	375	365	97	13
Codeine	225	240	107	7
	300	297	99	5
	375	407	108	9
Codeine glucuronide	225	241	107	10
	300	297	99	7
	375	388	104	16
Dihydrocodeine	225	251	111	9
	300	300	100	4
	375	405	108	13
6-AM	7.5	9	114	7
	10	10	97	5
	12.5	15	118	9
Pholcodine	225	246	109	5
	300	298	99	4
	375	388	104	9
Methadone	188	190	101	6
	250	243	97	3
	313	307	98	9
EDDP	188	183	97	10
	250	248	99	2
	313	284	91	12
Benzoylecgonine	113	123	110	7
	150	152	101	3
	188	213	114	11

Table 2.11 (cont.) – Drug screening assay: Inter-assay accuracy and precision data

Accuracy (% nominal QC values) and precision (% RSD)

Analyte	Nominal (µg/L)	Mean measured (µg/L)	Accuracy (%)	RSD (%)
Buprenorphine	3.25	4.0	106	9
	5.00	5.4	108	4
	6.75	6.3	100	9
Norbuprenorphine	3.25	3.9	103	7
	5.00	4.9	97	8
	6.75	6.5	104	10
Buprenorphine glucuronide	3.25	3.8	100	9
	5.00	5.1	103	8
	6.75	6.2	99	8
Norbuprenorphine glucuronide	3.25	3.9	103	10
	5.00	4.7	91	9
	6.75	6.1	98	8
Ketamine	38	41	108	8
	50	51	102	4
	63	61	97	11
Norketamine	38	38	102	6
	50	50	100	3
	63	60	95	10
Tramadol	150	146	97	8
	200	200	100	3
	250	233	93	11
O-Desmethyltramadol	150	170	113	7
	200	200	100	4
	250	259	104	9
N-Desmethyltramadol	150	172	114	13
	200	214	107	4
	250	265	106	11
6-Acetylcodeine	7.5	8	106	10
	10	10	96	6
	12.5	14	111	12
Noscapine	7.5	8	105	7
	10	9	90	4
	12.5	13	103	14
Papaverine	7.5	8	102	7
	10	9	92	7
	12.5	13	102	12

Table 2.12 – Drug screening assay: EQA accuracy data

Accuracy (% nominal concentration)

#LGC Proficiency Testing Drugs of Abuse in Urine Scheme

EQA Round#	Nominal concentration (µg/L)	Measured concentration (µg/L)	Accuracy (%)
Amfetamine			
108-1	178	188	105
109-3	1241	1040	84
110-1	567	535	94
111-3	1347	1333	99
113-1	1150	1182	103
115-1	1314	1302	99
Metamfetamine			
110-1	1238	1177	95
113-1	1423	1413	97
MDMA			
108-2	2879	2438	85
114-1	744	560	75
Mephedrone			
113-1	1272	1005	79
Benzoylecgonine			
108-1	1134	1095	97
108-3	105	122	117
109-2	492	472	96
110-1	668	638	96
111-2	718	692	96
112-1	728	680	93
112-3	76	87	114
113-1	665	661	99
113-3	371	327	88
Methadone			
108-1	2175	2481	114
108-3	234	272	116
110-1	1450	1450	100
112-2	590	605	103
113-3	583	539	92
115-2	609	618	101
EDDP			
108-1	2458	2350	96
108-3	239	232	97
110-1	884	947	107
112-2	529	490	93
Ketamine			
108-2	1700	1964	116
111-3	2583	2858	111
Tramadol			
114-1	18390	16557	90

Table 2.12 (cont.) – Drug screening assay: EQA accuracy data

Accuracy (% nominal concentration)

#LGC Proficiency Testing Drugs of Abuse in Urine Scheme

*Morphine is reported as 'total' morphine with a calculation used to convert morphine-3-glucuronide to its morphine equivalent. This is to reflect post-hydrolysis concentrations as most other laboratories do not directly measure glucuronides, but instead hydrolyse urine samples prior to analysis.

EQA Round#	Nominal concentration (µg/L)	Measured concentration (µg/L)	Accuracy (%)
Total Morphine*			
109-2	646	638	99
110-1	1480	1319	89
111-2	678	617	91
112-1	1369	1325	97
112-3	144	157	109
113-2	1472	1416	96
114-2	519	550	106
115-2	1404	1415	101
6-AM			
108-1	58	60	105
108-3	6	7	104
109-2	21	19	91
110-1	29	34	118
111-2	20	23	115
112-1	31	34	109
112-3	4	4	104
113-2	30	33	110
115-2	32	33	104
Buprenorphine			
108-1	8.0	8.2	103
112-2	7.8	7.6	97
113-1	7.5	7.9	105
114-3	353.2	327.7	93
Norbuprenorphine			
109-3	8.3	9.0	109

Table 2.13 – Drug screening assay: Summary of detection limits for each analyte and comparison to the selected cutoff concentration

Analyte	LoD (µg/L)	Cutoff (µg/L)
Amfetamine	13	200
Metamfetamine	50	200
MDMA	13	200
Mephedrone	13	200
Morphine	19	300
Morphine-3-glucuronide	38	300
Codeine	19	300
Codeine glucuronide	19	300
Dihydrocodeine	19	300
6-AM	5	10
Pholcodine	19	300
Methadone	63	250
EDDP	16	250
Benzoylecgonine	19	150
Buprenorphine	2.5	5
Norbuprenorphine	2.5	5
Buprenorphine glucuronide	2.5	5
Norbuprenorphine glucuronide	2.5	5
Ketamine	13	50
Norketamine	13	50
Tramadol	13	200
O-Desmethyltramadol	50	200
N-Desmethyltramadol	50	200
6-Acetylcodeine	2	10
Noscapine	3	10
Papaverine	3	10

Figure 2.17 – Drug screening assay: Extracted ion chromatograms of all analytes in the cutoff calibrator

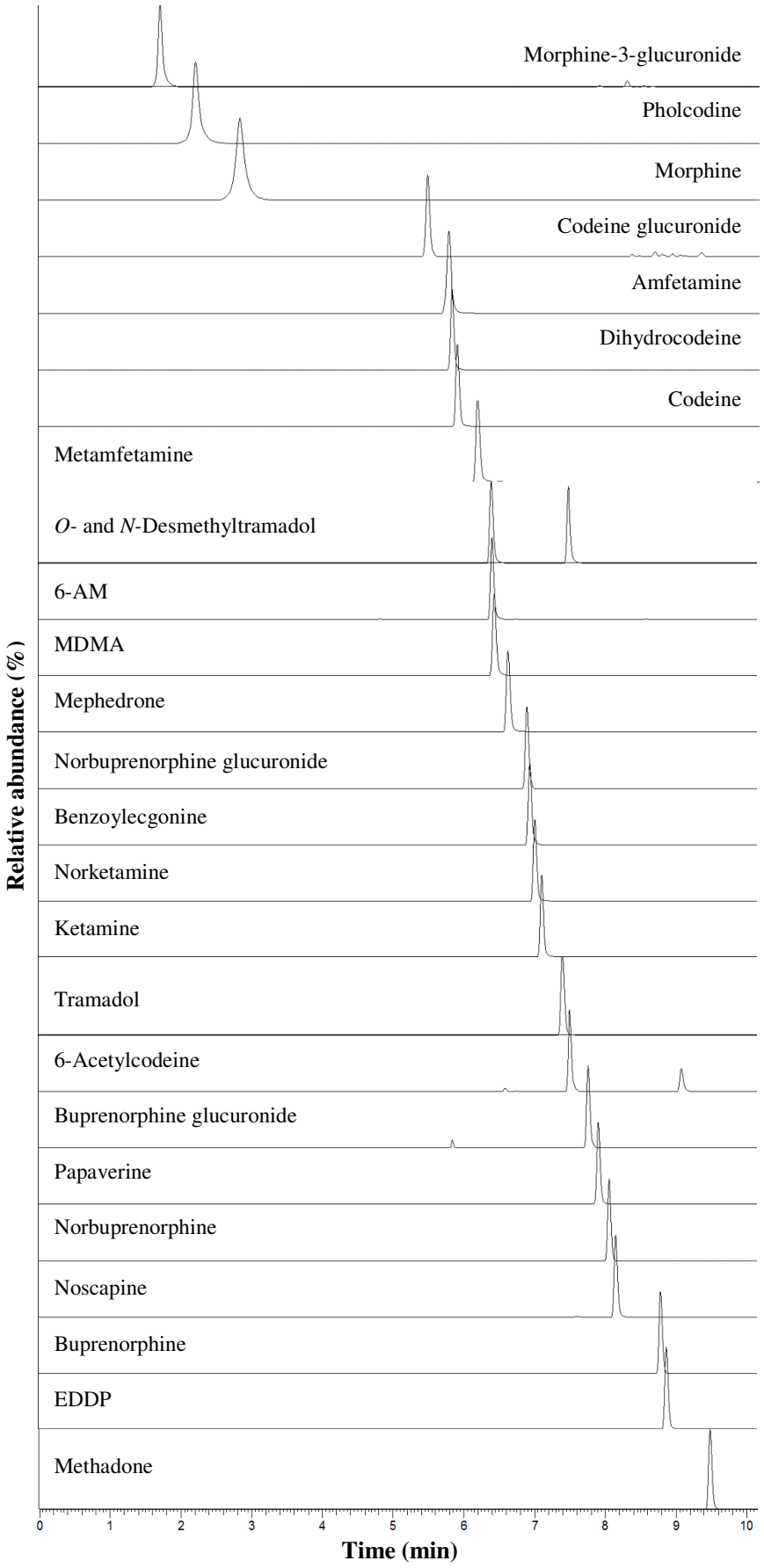


Table 2.14 – Drug screening assay: Typical mass accuracy for each analyte

The mean measured $[M+H]^+$ for each analyte was calculated from the analysis of calibrator and IQC solutions in sample batches

Analyte	Theoretical $[M+H]^+$ *(m/z)*	Within batch		Between batch	
		Mean Measured $[M+H]^+$ *(m/z)*	Mean *m/z* difference (ppm)	Mean Measured $[M+H]^+$ *(m/z)*	Mean *m/z* difference (ppm)
Amfetamine	136.1121	136.1119	-1.10	136.1118	-2.21
Metamfetamine	150.1277	150.1275	-1.74	150.1273	-2.76
MDMA	194.1176	194.1173	-1.22	194.1171	-2.35
Mephedrone	178.1226	178.1224	-1.39	178.1222	-2.57
Morphine	286.1438	286.1432	-2.16	286.1429	-3.21
Morphine-3-glucuronide	462.1759	462.1750	-2.04	462.1744	-3.20
Codeine	300.1594	300.1588	-2.17	300.1585	-3.14
Codeine glucuronide	476.1915	476.1908	-1.60	476.1903	-2.57
Dihydrocodeine	302.1751	302.1744	-2.33	302.1741	-3.23
6-AM	328.1543	328.1536	-2.14	328.1533	-3.29
Pholcodine	399.2278	399.2271	-1.96	399.2266	-3.17
Methadone	310.2165	310.2158	-2.31	310.2156	-3.20
EDDP	278.1903	278.1897	-2.27	278.1894	-3.41
Benzoylecgonine	290.1387	290.1381	-2.10	290.1378	-3.12
Buprenorphine	468.3108	468.3101	-1.52	368.3097	-2.55
Norbuprenorphine	414.2639	414.2629	-2.24	414.2625	-3.23
Buprenorphine glucuronide	644.3429	644.3420	-1.61	644.3413	-2.65
Norbuprenorphine glucuronide	590.2960	590.2953	-1.21	590.2947	-2.26
Ketamine	238.0993	238.0990	-1.29	238.0987	-2.46
Norketamine	224.0837	224.0833	-1.73	224.0831	-2.70
Tramadol	264.1958	264.1954	-1.83	264.1950	-3.00
O-Desmethyltramadol	250.1802	250.1796	-2.35	250.1793	-3.49
N-Desmethyltramadol	250.1802	250.1796	-2.41	250.1793	-3.59
6-Acetylcodeine	342.1700	342.1693	-2.07	342.1689	-3.33
Noscapine	414.1547	414.1539	-2.08	414.1535	-3.14
Papaverine	340.1543	340.1542	-0.58	340.1537	-1.89

Table 2.15 – Drug screening assay: Summary matrix effects data

Solutions containing all analytes at the relevant cutoff concentration were prepared in (i) analyte-free human urine from 20 independent sources, and (ii) eluent A. Prepared solutions were diluted (1+9, v/v) with eluent A and analysed. The peak area of each analyte/internal standard was compared in the presence and absence of matrix to calculate the matrix effect.

Analyte	Mean Matrix Effect (%)	Relative Matrix Effect (%)
Amfetamine-$^{13}C_6$	79	-
Amfetamine	78	101
Metamfetamine-D_{14}	40	-
Metamfetamine	52	130
MDMA-D_5	44	-
MDMA	45	102
Mephedrone-D_3	39	-
Mephedrone	31	79
Morphine-D_3	46	-
Morphine	46	100
Morphine-3-glucuronide	31	67
Pholcodine	25	54
Codeine-D_6	50	-
Codeine	46	92
Codeine glucuronide	73	146
Dihydrocodeine-D_6	44	-
Dihydrocodeine	46	105
Methadone-D_3	79	-
Methadone	84	106
EDDP	80	101
Benzoylecgonine-D_3	75	-
Benzoylecgonine	77	103
6-AM-D_3	67	-
6-AM	66	99
6-Acetylcodeine	66	99
Noscapine	52	78
Papaverine	67	100
Buprenorphine-D_4	104	-
Buprenorphine	103	99
Buprenorphine glucuronide	129	124
Norbuprenorphine-D_3	62	-
Norbuprenorphine	75	121
Norbuprenorphine glucuronide	73	118
Ketamine-D_4	56	-
Ketamine	64	114
Norketamine-^{13}C	68	-
Norketamine	67	101
Tramadol-^{13}C-D_3	64	-
Tramadol	70	109
N-Desmethyltramadol	64	100
O-Desmethyltramadol	68	106

2.6.1 Isobaric Interferences

Mass spectrometry is a highly selective method, but it has limitations. Interference may arise from isobaric analytes, for example. To overcome this, compounds should be resolved chromatographically and/or produce different MS^2 fragments in order to correctly identify the compound present. Known isobaric interferences were investigated to ensure the compound of interest could be distinguished from its isobar/s.

Complete LC resolution was achieved for the isobaric compounds tested, and different MS^2 product ions were also identified in each case to aid compound identification (Table 2.16).

Table 2.16 – Drug screening assay: Chromatographic resolution and MS product ion spectra for some isobaric substances

[#] Ions resulting from a water loss excluded

Analyte	Molecular formula	Precursor ion (*m/z*)	Dominant product ion/s[#] (*m/z*)	Retention time (min)
Morphine	$C_{17}H_{19}NO_3$	286.1438	201.0910 181.0648	2.87
Norcodeine			225.0910 121.0648	5.75
Norhydrocodone			199.0755 241.0860	6.30
Hydromorphone			185.0597 157.0648	4.42
Hydrocodone	$C_{18}H_{21}NO_3$	300.1594	157.0648 185.0597	6.38
Codeine			215.1067 199.0754 165.0670	5.89
Noroxycodone	$C_{17}H_{19}NO_4$	302.1387	187.0753 227.0937	6.15
Oxymorphone			227.0941 198.0913	3.29
6-AM	$C_{19}H_{21}NO_4$	328.1543	165.0696 211.0750 193.0643	6.30
Naloxone			212.0701 253.1089	5.72
EDDP	$C_{20}H_{23}N$	278.1903	234.1270 249.1507 186.1273	8.98
Amitriptyline			91.0546 105.0701 117.0700	9.34
Tramadol	$C_{16}H_{25}NO_2$	264.1958	58.0644	7.18
N-Desmethylvenlafaxine			215.1430	6.81
O-Desmethylvenlafaxine			215.1430	7.90
N-Desmethyltramadol	$C_{15}H_{23}NO_2$	250.1802	189.1274	7.49
O-Desmethyltramadol			58.0651	6.39
N,O-Didesmethylvenlafaxine			215.1430	6.88

2.7 Patient Sample Comparison

There were 500 urine samples from 343 individuals. Demographic information was available for 373 samples. Of these, 251 (67 %) were from males and 122 (33 %) from females. The median (range) age at the time of sampling was 40 years (0-78). Patient results are summarised in Table 2.17. Differences in results between the two methods were largely attributed to either false-positive results by immunoassay, or different analyte cutoff concentrations being used for the two methods.

2.7.1 Immunoassay False-Positives

The buprenorphine immunoassay has low specificity, with the highest number of false-positives associated with this assay. Fifty-two samples (10.4 %) were classed as positive by immunoassay but neither buprenorphine nor its metabolites were detected by LC-HRMS. The immunoassay is prone to interference from high opioid concentration within a urine sample (Pavlic *et al.*, 2005). Forty-eight of these samples had a CEDIA opioid concentration >2000 µg/L. In the remaining samples, other drugs known to interfere with the immunoassay were detected (amisulpride N = 2, nalbuphine N = 1, and trimethoprim N = 1).

As previously discussed, the amfetamine immunoassay is prone to interference from many therapeutic drugs. Eight samples (1.6 %) were classed as negative by LC-HRMS (i.e. amfetamine, metamfetamine, MDMA, and mephedrone not detected). Ranitidine (N = 2), mebeverine (N = 2), atomoxetine (N = 2) and ephedrine (N = 1) were identified in the samples which likely accounts for the positive amfetamine immunoassay result due to structural similarity to metamfetamine, the compound the immunoassay is calibrated against (Figure 2.18). No known interfering analytes were detected in the remaining sample.

Table 2.17 – Contingency tables summarising patient results when using LC-HRMS versus immunoassay for drug screening

LC-HRMS was taken as the reference method, with immunoassay sensitivity and selectivity calculated according to the equations below

$$Sensitivity = \frac{True\ Positives}{(True\ Positives + False\ Negatives)} \times 100$$

$$Specificity = \frac{True\ Negatives}{(True\ Negatives + False\ Positives)} \times 100$$

Amfetamines
Sensitivity 95 % Specificity 98 %

	LC-HRMS Positive	LC-HRMS Negative
Immunoassay Positive	19	8
Immunoassay Negative	1	472

Cocaine (as benzoylecgonine)
Sensitivity 94 % Specificity 100 %

	LC-HRMS Positive	LC-HRMS Negative
Immunoassay Positive	151	0
Immunoassay Negative	10	339

Methadone Metabolite (EDDP)
Sensitivity 100 % Specificity 98 %

	LC-HRMS Positive	LC-HRMS Negative
Immunoassay Positive	187	6
Immunoassay Negative	0	307

Opioids
Sensitivity 100 % Specificity 96 %

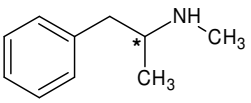
	LC-HRMS Positive	LC-HRMS Negative
Immunoassay Positive	207	11
Immunoassay Negative	0	282

Buprenorphine and metabolites
Sensitivity 99 % Specificity 87 %

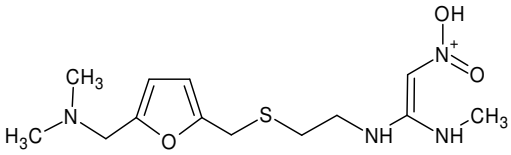
	LC-HRMS Positive	LC-HRMS Negative
Immunoassay Positive	98	52
Immunoassay Negative	1	349

<u>Figure 2.18</u> – Comparison of the structures of ranitidine, mebeverine, ephedrine, and atomoxetine to metamfetamine

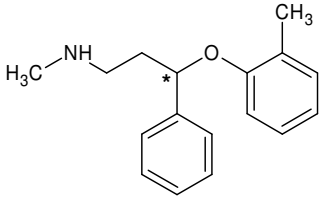
* chiral centre



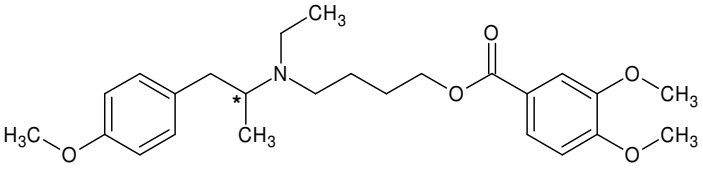
Metamfetamine



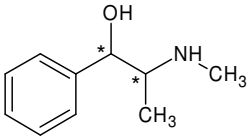
Ranitidine



Atomoxetine



Mebeverine



Ephedrine

2.7.2 Immunoassay False-Negatives

One false-negative was observed for buprenorphine. In this sample, only norbuprenorphine glucuronide was detected by LC-HRMS. Norbuprenorphine glucuronide does not cross-react when analysed by CEDIA (<0.015 % cross reactivity, ThermoFisher Scientific, 2016) and thus would explain the negative result.

The amfetamine-group CEDIA is calibrated using *S*-metamfetamine, with *S/R*-amfetamine having 88 % of the cross-reactivity of metamfetamine (ThermoFisher Scientific, 2014). One sample had a calculated amfetamine concentration of 716 µg/L by LC-HRMS and 470 µg/L by CEDIA, which may relate to the reduced cross-reactivity of amfetamine with the immunoassay.

2.7.3 Differences in Analyte Cutoff Concentration

The use of different cutoff concentrations between CEDIA and the LC-HRMS methods account for the apparent differences in the cocaine and methadone results.

A lower cutoff for benzoylecgonine was selected for the LC-HRMS method to be in-line with EWDTS guidelines (150 µg/L) than was used for the CEDIA (300 µg/L). If the CEDIA cutoff was decreased to 150 µg/L only 1 result differed (CEDIA: 116 µg/L, LC-HRMS: 160 µg/L).

A higher cutoff for EDDP was selected for the LC-HRMS method to be in-line with EWDTS guidelines (250 µg/L) than was used for the CEDIA (100 µg/L). If the CEDIA cutoff concentration was increased to 250 µg/L only 2 results differed (CEDIA: 256 and 385 µg/L, and LC-HRMS: 207 and 240 µg/L, respectively).

2.7.4 Additional Immunoassay Cross-reactivity

For the opioids, the calculated immunoassay concentration is a composite result derived from all opioids in the sample cross-reacting to a greater or lesser extent, whereas the LC-HRMS method measures selected opioids. The 11 samples which were opioid negative by LC-HRMS all had a CEDIA concentration of <750 µg/L. Morphine was detected by LC-HRMS in all samples, but at a concentration below the cutoff concentration, with total morphine ranging from 166-287 µg/L. Minor metabolites of morphine (e.g. normorphine, hydromorphone) which are not targeted using the LC-HRMS method may cross-react with the immunoassay method and account for the higher observed opioid concentration.

2.7.5 Analytes without Immunoassay Available

No immunoassay was used in the laboratory for the detection of tramadol or ketamine. Within the 500 patient urine samples analysed as part of the routine drug screening cross-validation, tramadol (and its metabolites) was detected in 14 samples (2.8 %), and ketamine (and norketamine) in 4 samples (0.8 %).

2.8 Conclusions

An LC-HRMS method has been developed and validated that is suitable for clinical drug detection. The use of LC-HRMS as a drug screening method negates the need for prior immunoassay analysis for most drug classes, reducing cost and turn-around-times. A compromise had to be drawn with analysis of some analytes remaining by immunoassay to offer a practical method in the clinical laboratory. A similar 'hybrid' approach has been adopted by a clinical laboratory in America (McMillin *et al.*, 2015).

The use of LC-HRMS for routine drug detection in a clinical setting has two key advantages; retrospective data analysis and rapid incorporation of new analytes into the method. The developed method offers a novel approach to clinical drug analysis by enabling specific drugs of abuse to be targeted as well as providing the capacity to detect other analytes through the collection of full scan MS data. This enables retrospective data analysis without the need to re-analyse the sample if the presence of additional compounds is queried at a later stage. The amount of data produced for each sample is large, and thus storage of data is an important consideration to ensure data are available for such retrospective work. The method is easily adaptable to include other analytes that may be required in a routine urine drug screen. Since initial development of the method, methylphenidate, ethylphenidate, and ritalinic acid have been added as additional analytes (Chapter 4). The versatility of the assay allows new drugs of abuse to be incorporated into the method without detriment to the original panel of analytes. Improving detection of NPS is of increasing importance to enable accurate diagnosis and treatment of individuals who abuse these substances.

The decision to use immunoassay for selected drugs was largely based on analytical challenges. Barbiturates are not ionized using positive-mode MS and the time taken to switch between ionisation modes resulted in too few data points across chromatographic peaks. For cannabis there was an issue with assay sensitivity. The immunoassays for cannabis and barbiturates are more selective than many of the other drug immunoassays, and thus false positives are unlikely. In addition, the number of samples screening positive for barbiturates has fallen over time and it is likely that most samples will not contain these analytes. As barbiturates are rarely encountered, whether they should be included in a routine drug screen is questionable. It may be more appropriate to offer barbiturates as an additional test; this will be explored in Chapter 6 when auditing clinical data. For the benzodiazepine class of drugs, a large number of analytes would need to be included to offer a comparative result to the CEDIA. This was not

deemed practical for a routine clinical service as there was not clinical justification for the time and cost implications. The benzodiazepine immunoassay is not prone to false positives, but may give negative results for more potent, short-acting benzodiazepines such as clonazepam. Note finally that if a false positive or negative cannabis or benzodiazepine immunoassay result is queried, retrospective interrogation of the LC-HRMS data can be conducted to confirm the presence or absence of benzodiazepines and THC derivatives.

2.8.1 Further Work

The work presented here highlights some of the challenges facing comprehensive screening for drugs of abuse in a routine clinical setting. The main issues relate to the huge amount of data generated when using HRMS and the lack of software to reliably process it. If inclusion of new analytes is to be undertaken, this should be driven by clinical requirement. Currently there is interest in the detection of synthetic cannabinoids, although due to the sheer number of these compounds and their likely low concentration in urine a separate method may be needed.

Incorporation of cannabis detection into the LC-HRMS method through improving assay sensitivity should be further investigated. As THC-derivatives elute away from the majority of analytes, inclusion of a different MS scan experiment may be possible without impacting other analytes. A targeted MS scan, such as selected ion monitoring (SIM) may improve sensitivity.

Currently the developed method is only validated for urine samples. Validation of the method for oral fluid could be investigated as there is clinical interest in utilising this matrix to avoid problems associated with patients adulterating urine samples. As oral fluid is a more complex matrix than urine, development of a suitable sample preparation method would be needed. In addition, the collection device to use would need to be evaluated to ensure good recovery of the analytes of interest.

3 Qualitative Drug Analysis by LC-HRMS: Application to identification of unknown substances in biological and non-biological samples

3.1 Introduction

3.1.1 The Purpose of Unknown Screening

Identifying drugs present in substances such as tablets or powders found on a patient via toxicological analyses may aid clinicians in the diagnosis of poisoning, particularly if the patient is unconscious. In some cases, unknown substance screening not only enlightens clinicians as to what has actually been consumed, but also informs the individual themselves. Drug users may take a substance believing it is a one drug when in actual fact it may be a different compound entirely; this particularly applies to NPS that are constantly changing. In addition, it may not be the actual drug that is causing adverse clinical features, but other substances (adulterants) present within the powder or pill being taken; again the user is often unaware that these compounds are being consumed.

Analysis of a biological sample, such as urine, is necessary to actually confirm that an individual has taken a substance. Drug identification is more challenging in urine than in the substance itself as (i) potent drugs may be present at very low concentration, (ii) the parent drug may not be present due to metabolism, and in some cases metabolites may not be well documented, and (iii) endogenous compounds will be present that may complicate the analysis.

3.1.2 Drug Identification

Many tablets and capsules have no discerning features and cannot be identified reliably by sight alone. In some cases, even labelled tablets may not contain the expected compounds. For example, the brand name of NPS products often remain the same, but the constituents change. Analysis of NPS products has shown that the stated contents frequently do not match the compounds actually present in the product (Ford and Berg, 2017). Similarly, synthetic cannabinoids, which are typically sprayed onto inert plant material to mimic the appearance of traditional cannabis, are visually indistinguishable from tobacco. Unlike cannabis, synthetic cannabinoids do not possess a distinctive smell. As a result, identification of synthetic cannabinoids can only be performed via chemical analysis.

3.1.3 Detection of Adulterants

3.1.3.1 Herbal Medicines and Dietary Supplements

Despite herbal medicine not being incorporated into Western primary healthcare, purchase of herbal medicines over-the-counter (OTC), or over the internet, is increasing with the trend toward use for 'general well-being'. Many individuals omit to mention herbal medicines when asked to disclose medications they are taking, which can make it challenging for clinicians to discern the cause of a change in health or in concentrations of prescribed drugs due to pharmacokinetic interactions. These products suffer from a lack of regulation and standardisation, meaning the user may be unaware of what they are actually consuming. Whilst labelled as 'natural', the apparent efficacy of a herbal preparation may indeed be due to the presence of synthetic pharmaceutical agents as opposed to any herbal component. Herbal remedies marketed for erectile dysfunction have been found to contain sildenafil and/or sildenafil analogues such as tadalafil and homosildenafil, for example. The US FDA Forensic Center analysed 40 dietary supplements advertised as sexual enhancers and found that 19 contained synthetic drugs (Gratz *et al*., 2004). Other than licensed pharmaceutical drugs, synthetic drugs that have been banned due to associated health risks have also been detected in herbal products. Herbal slimming formulas in many countries, including the UK, Australia, and Canada, have been found to contain sibutramine, fenfluramine, and phenolphthalein, for example (Calahan *et al.*, 2016; Yen and Ewald, 2012).

3.1.3.2 Illicit Drugs

The purity of illicit drugs varies significantly, with most 'cut' through the addition of adulterants to increase their weight or alter their physical properties. Cutting agents are generally either inert bulking agents, or pharmaceutically active ingredients; the latter may enable greater dilution of the drug without the user perceiving any quality reduction due to mimicking of the illicit drugs effects (e.g. benzocaine in cocaine powders). Variation in purity between drug batches is problematic at two levels; firstly because users may not modify the quantities used and could consume more drug than intended leading to acute toxicity, and secondly the adulterants added may have direct toxicity. Detection of adulterants present in a drug may aid diagnosis of a clinical condition. Commonly used bulking agents include boric acid, lactose, mannitol and creatine, which typically have low toxicity. Pharmaceutically active cutting agents are of greater concern as there may be harm associated with their use. For example, levamisole present in cocaine powders can cause agranulocytosis and skin necrosis

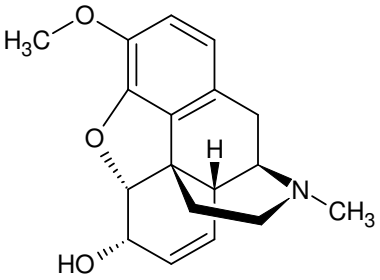
(Larocque and Hoffman, 2012). Currently in Europe, the main adulterants found in heroin are paracetamol and caffeine, and phenacetin, levamisole, caffeine, diltiazem, hydroxyzine and lidocaine are commonly found in cocaine (Broséus *et al.*, 2016).

The presence of NPS as adulterants in cocaine powders has also been reported, albeit infrequently. In 2012, methylone was identified in 1.65 % of MDMA tablets analysed by a drug checking service in Switzerland; and methylone, 4-methylethcathinone (4-MEC), and mephedrone were identified in 8.8 %, 10.2 %, and 9.5 %, respectively, of MDMA tablets analysed by the drug checking service 'Checkit!' in Austria (Giné *et al.*, 2014).

3.1.4 The Use of HRMS for Unknown Compound Identification

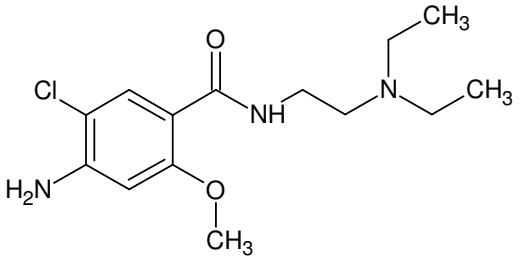
The main justification for using HRMS is that by measuring accurate masses of analytes putative molecular formulae for an unknown compound can be calculated. This is not possible when using nominal mass spectrometers as different molecular formula cannot be distinguished. For example, codeine and metoclopramide both have a nominal mass of 300.1 amu, but their exact masses are distinct, 300.1594 and 300.1473 respectively, due to different elemental compositions (Figure 3.1).

<u>Figure 3.1</u> – Structures of codeine and metoclopramide



CODEINE
$C_{18}H_{21}NO_3$

nominal mass = 300.1 amu
exact mass = 300.1594 amu



METOCLOPRAMIDE
$C_{14}H_{22}ClN_3O_2$

nominal mass = 300.1 amu
exact mass = 300.1473 amu

Another advantage of using HRMS is that unlike triple quadrupole instruments, the masses of analytes do not have to be selected prior to analysis. Thus, when using targeted methods for unknown drug screening, only drugs from a pre-defined list will be screened for. Other drugs which may be present will not be detected and this could lead to reporting of false negative results. Due to dwell time and time segment restrictions, selected reaction monitoring methods generally include a limited number of analytes to ensure enough data points are collected over chromatographic peaks.

3.1.5 Aim

This chapter explores the use of the LC-HRMS method developed (Chapter 2) for identifying drugs in biological and in non-biological samples and aims to assess whether the method is suitable for routine clinical use.

3.2 Methods

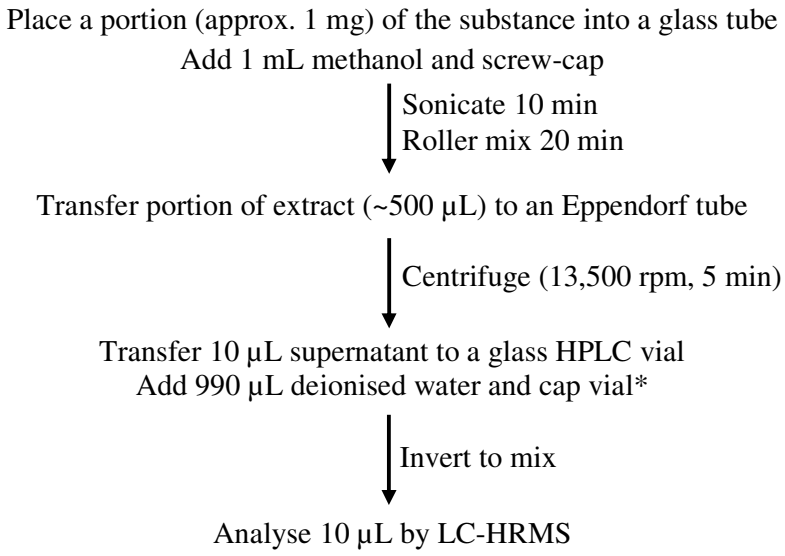
3.2.1 Sample Preparation

Sample preparation depends upon the nature of the sample to be analysed, but can broadly be divided into whether it is biological or not. Typical protocols used for each sample type are outlined below.

3.2.1.1 Solid Substances

All physical features of the solid material were recorded, particularly for tablets, including the size, shape, colour, and any markings present. The sample was extracted with methanol (Figure 3.2), with tablets being crushed using a pestle and mortar prior to the extraction.

Figure 3.2 – Sample extraction protocol for solid unknown substances



*If precipitation of a component in the substance occurs, centrifuge (13,500 rpm, 5 min) and transfer supernatant to a clean HPLC vial for analysis.

3.2.1.2 Urine Samples

Samples were prepared as described in Section 2.2.9.

3.2.2 Analytical Method

The LC-HRMS method (Chapter 2) was used for the analysis of all unknown substances.

3.2.3 Resources

The main resources used to aid substance identification are described below.

3.2.3.1 Qual Browser

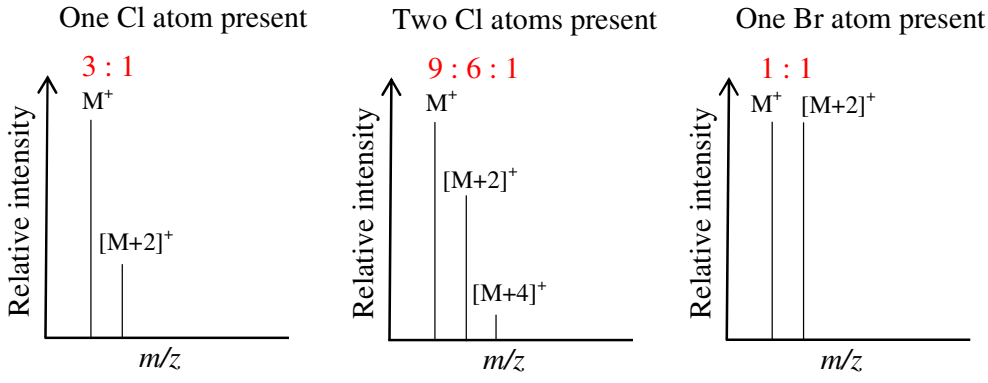
Qual Browser is part of the Xcalibur™ software provided by ThermoFisher Scientific. Qual Browser allows raw data to be viewed both as chromatograms and mass spectra. Chromatographic data can be viewed as either the total ion chromatogram (TIC), or as an extracted ion chromatogram (XIC). For identifying unknown substances, the TIC is examined to identify any chromatographic peaks in the sample analysis. From this, the observed *m/z* at a specific time, or over a time range, can be ascertained to identify the precursor ion/s present. Similarly, product ions may be identified from MS^2 data.

Studying the mass spectrum to identify the isotope pattern of a precursor ion may provide extra information about the structure of an analyte. For instance, if a chlorine atom is present in the structure a mass peak 2 amu above the precursor ion (M+2) will be apparent due to the natural occurrence of ^{37}Cl. The ratio of the two resulting mass peaks will be approximately 3:1 (the natural abundance of ^{35}Cl to ^{37}Cl 75:25). In a similar manner, if the compound contains two chlorine atoms a mass peak 4 units above the precursor ion (M+4) will be apparent in addition to the M+2 peak. The presence of bromine in a molecule can also be identified by the presence of an M+2 peak; however, the ratio of the M+ and M+2 peaks will be approximately 1:1 (the natural abundance of ^{79}Br and ^{81}Br 50.5:49.5, Figure 3.3).

Possible molecular formulae can be generated by the software for any *m/z* present in the mass spectrum ($z = 1$ assumed), with the mass deviation of the proposed structure from the observed *m/z* given in milli mass units (mmu).

Figure 3.3 – Simplified graphs to show the relative intensities of the molecular ion and its major isotope peaks when chlorine and bromine are present in a structure

Peak ratios are shown in red.



3.2.3.2 ChemSpider

ChemSpider is an on-line database (www.chemspider.com) provided by the Royal Society of Chemistry containing over 58 million chemical structures. For compound identification, a search against molecular formulae can be conducted to list compounds with this structure.

When conducting a ChemSpider search, the structure generated by Xcalibur needs to either have a hydrogen atom removed or added to account for ionisation of the compound in positive or negative ion mode, respectively.

3.2.3.3 *mz*Cloud

*mz*Cloud is an on-line database (www.mzcloud.org) provided by HighChem LLC (Bratislava, Slovakia) with many partners including universities across the world and instrument manufacturer ThermoFisher Scientific. The database is constantly evolving with new compounds being added; currently 7,055 compounds are described (accessed 21/11/2017). The database comprises high-resolution mass spectra with each record containing compound name, synonyms, chemical structure, precursor *m/z*, and raw mass spectra for MS/MS or multi-stage MSn. For each mass spectrum, information such as the CID and HCD are included to aid the analyst to choose data that are comparable to the method being used. Searches can be conducted using compound name, monoisotopic mass and chemical structure.

3.2.3.4 TICTAC

TICTAC Communications Ltd. is a leading provider of drug identification and other information to the criminal justice and healthcare sectors, and is based at St George's University of London (www.tictac.org.uk). As part of their service, a database providing visual drug identification for solid drugs (tablets, capsules, patches and stamps) has been established that currently contains over 32,700 medicines, drugs and products that may be confused with drugs. Searches are based upon the physical characteristics of a drug, for example colour, size, score marks, and any images or text present. Results are then returned that match the criteria entered, listing the compounds identified in these products.

3.2.4 Identification Criteria

To improve the reliability and confidence in identification of a compound, as many identification criteria as possible should be used. To be certain of analyte identification, a reference material for the compound is required to enable a direct comparison with the results of sample analysis, particularly for retention time and product ions. Where a reference standard is not available, only tentative identification is possible.

3.2.4.1 Retention Time

Retention time is one of the most important identification criteria; however it is only useful if a reference material is available for the compound of interest. For NPS, often a reference compound is not commercially available or may be too costly to purchase.

3.2.4.2 Monoisotopic Molecular Mass

Accurate mass reduces the number of possible analytes as compared to using nominal mass spectrometers; however, compound identification based on accurate mass alone is insufficient (Remane *et al*., 2016). A mass error of <5 ppm is accepted for comparison of an observed *m/z* to the theoretical *m/z*.

3.2.4.3 Product Ions

Where possible, structure-specific ions should be used to confirm an analytes identity as opposed to non-specific losses (e.g. water loss equates to a product ion 18 amu less than the precursor ion). Identifying a greater number of product ions can increase selectivity.

3.2.4.4 Ionisation Mode

Some analytes will only ionise either in positive, or in negative ion mode. However, there are some analytes that will ionise in both modes; in this case identification of the compound can be enhanced through the presence of a chromatographic peak at the same retention time in both the positive and negative ion scans.

3.2.4.5 Isotope Pattern

As mentioned in Section 3.2.3.1, the presence of an M+2 peak can act to confirm the presence of a halogen atom (typically chlorine in the case of drugs) within a structure. This can reduce the potential number of molecular formula matches for a given *m/z*.

3.2.4.6 Metabolites

Identification of metabolites in urine samples is important when confirming exposure to a compound. Moreover, the parent drug may not always be present in urine, particularly if the drug undergoes extensive metabolism, or if drug intake was not recent.

3.3 Method Application to Case Studies

3.3.1 A 'Diet Pill' Purchased from the Internet

A 24-year-old female bought 'diet pills' from uncertified sources and presented to her general practitioner with clinical features of toxicity that included nausea, headaches, tremor and severe constipation. A single white and green capsule was submitted for analysis (Figure 3.4). The TIC showed a large peak at 9.52 min with some smaller peaks between 8-11 min (Figure 3.5). The observed *m/z* at 9.52 min was 280.1819, and a single chlorine atom was identified in the compound through the presence of the M+2 peak (Figure 3.6). Another chromatographic peak at 9.05 min was identified, with *m/z* 319.0957. A literature search was conducted to identify compounds that had been detected in counterfeit diet pills to guide further investigation (Table 3.1).

The observed *m/z* for the peak at 9.52 min was close to the theoretical *m/z* for sibutramine (mass error -2.86 ppm), and the observed *m/z* for the peak at 9.05 min close to the theoretical *m/z* for phenolphthalein (mass error -2.51 ppm). In addition, sibutramine contains a single chlorine atom in its structure which fitted the observation of the M+2 peak seen at 9.52 min. Product ions for sibutramine (139.0305, 125.0151, 127.0120) and phenolphthalein (225.0546, 197.0597, 105.0335) were identified through analysis of a reference solution (Sigma Aldrich) and from *mz*Cloud, respectively. All product ions were present in the relevant MS^2 spectra, confirming the presence of sibutramine and phenolphthalein in the capsule contents (Figure 3.7).

Sibutramine is a serotonin noradrenaline reuptake inhibitor (SNRI) which has been used in the treatment of obesity. In recent years, the market for dietary supplements has grown and many products are available over the internet. Both sibutramine and phenolphthalein have been reported as commonly detected components of many different weight-loss tablets (Tang *et al.*, 2011). However, many countries including the UK have withdrawn the drug from use due to concerns over potential adverse cardiovascular side effects such as hypertension, tachycardia, myocardial infarction (Azarisman *et al.*, 2007). Phenolphthalein has been used as a laxative, but the drug has also been withdrawn due to concerns over carcinogenicity.

Figure 3.4 – Physical appearance of a) the capsule, and b) the contents within the capsule

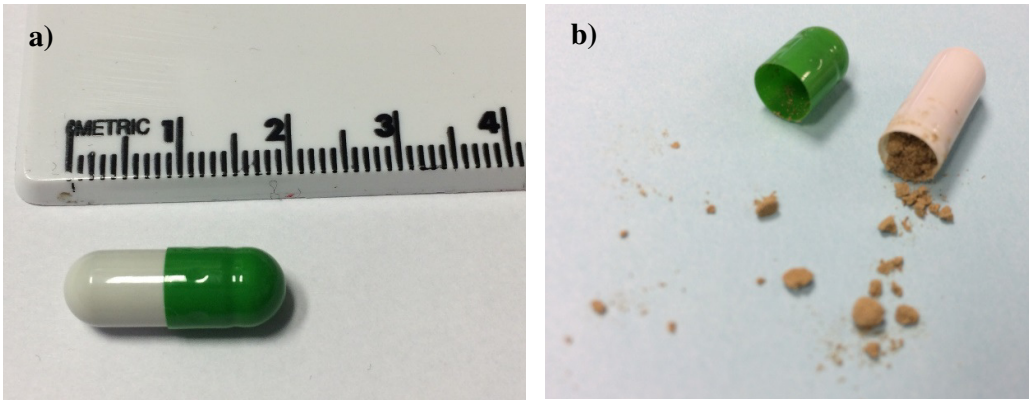


Table 3.1 – Reported substances identified in counterfeit diet pills (source data: Tang *et al.*, 2011; Yen and Ewald, 2012)

Compound	Molecular Formula	$[M+H]^+$ (*m/z*)
Sibutramine	$C_{17}H_{26}ClN$	280.1827
Benfluorex	$C_{19}H_{20}F_3NO_2$	352.1519
Fenfluramine	$C_{12}H_{16}F_3N$	232.1308
Phenolphthalein	$C_{20}H_{14}O_4$	319.0965
Bisacodyl	$C_{22}H_{19}NO_4$	362.1387
Phentermine	$C_{10}H_{15}N$	150.1277
Aminorex	$C_9H_{10}N_2O$	163.0866
L-Carnitine	$C_7H_{15}NO_3$	162.1125
Chlorogenic acid (green coffee extract)	$C_{16}H_{18}O_9$	355.1024
Epigallocatechin (green tea extract)	$C_{15}H_{14}O_7$	307.0812
Caffeine	$C_8H_{10}N_4O_2$	195.0877

Figure 3.5 – Analysis of the capsule extract showing a) the TIC, b) an XIC for *m/z* 280.1819, and c) an XIC for *m/z* 319.0957 (see Section 3.3.1)

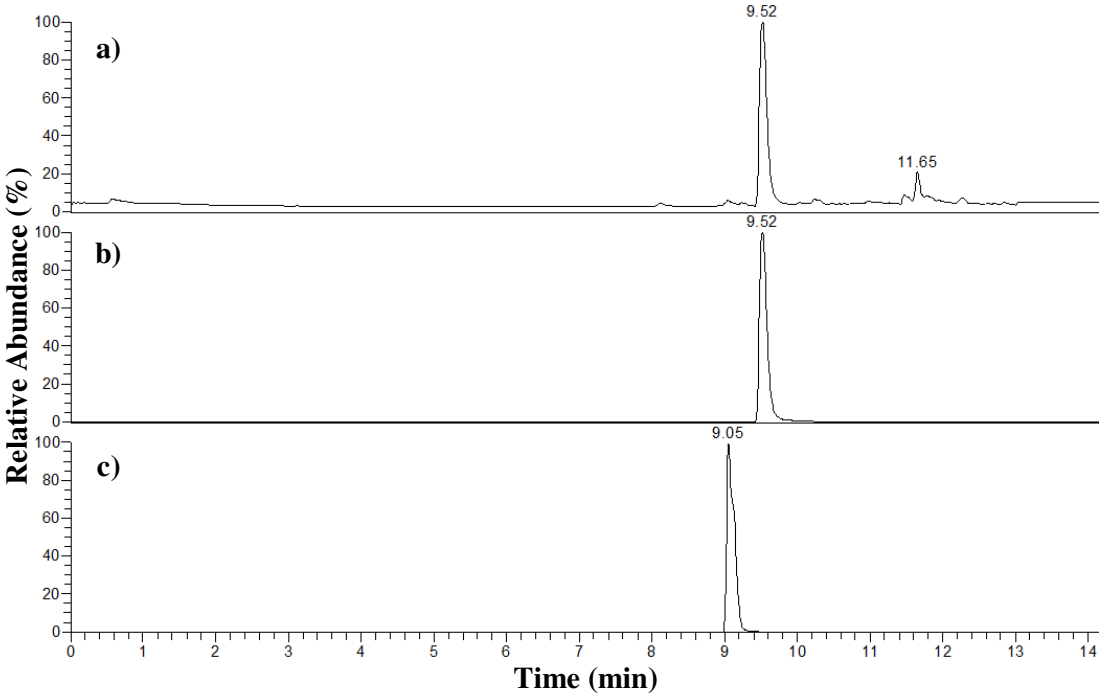


Figure 3.6 – Mass spectra of the precursor ions for the compounds eluting at a) 9.52 min, and b) 9.05 min (see Figure 3.5)

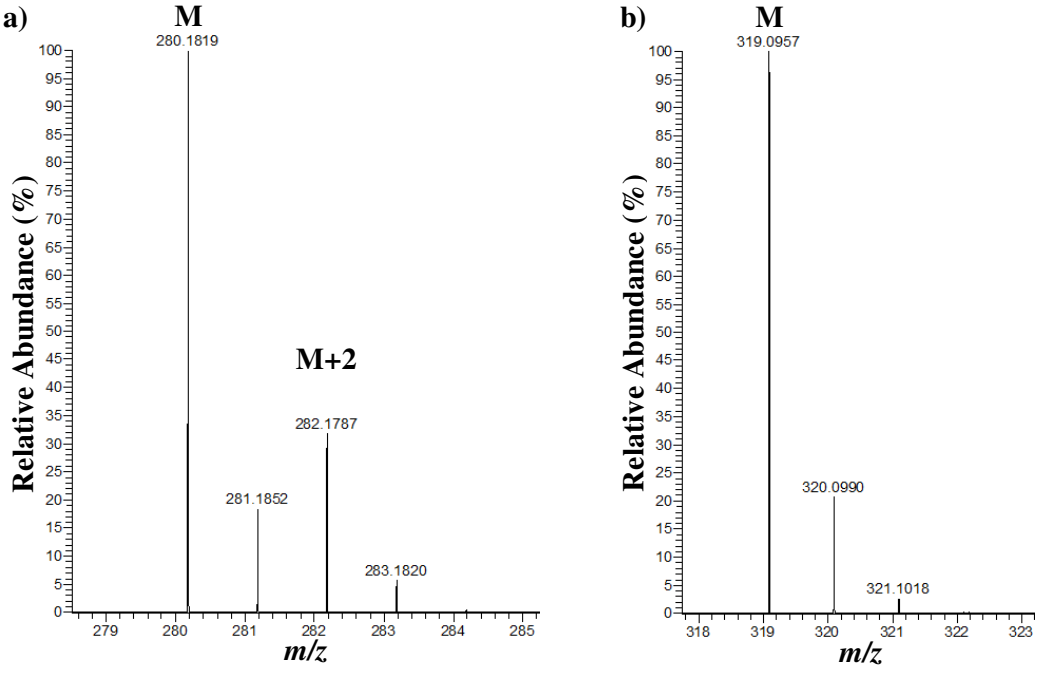
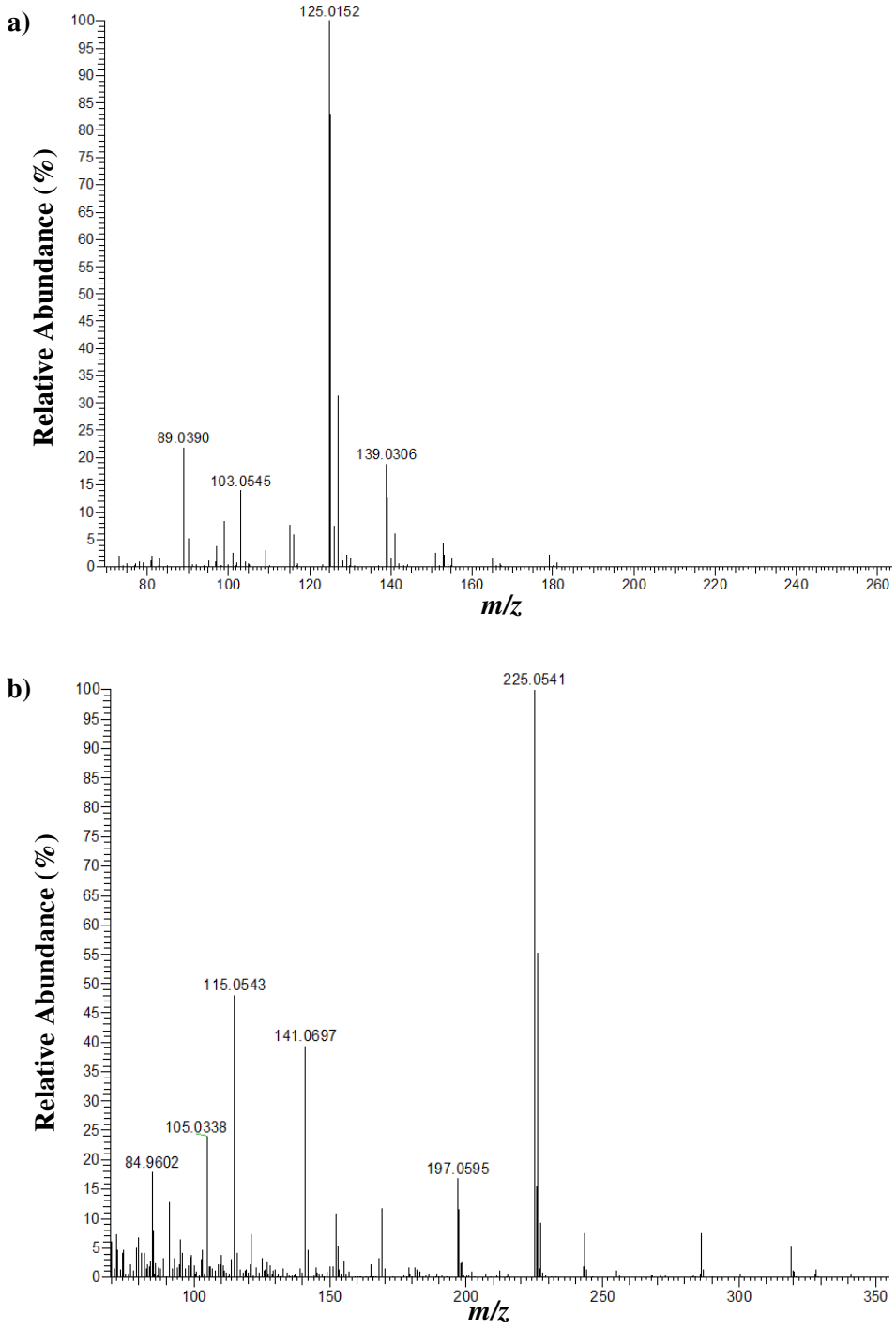


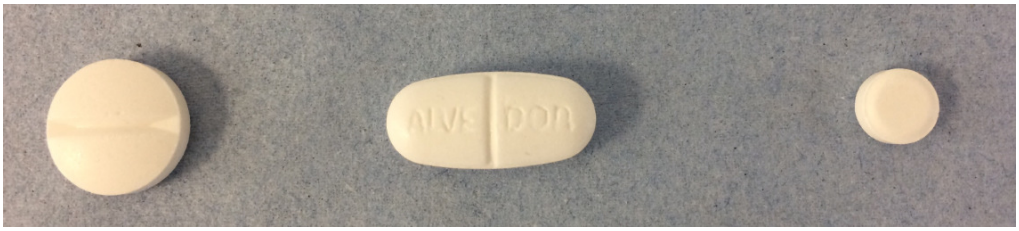
Figure 3.7 – MS^2 spectra to show the product ions present at a) 9.52 min, and b) 9.05 min (see Figure 3.5)



3.3.2 Tablets Found on an Individual on Admission to Hospital

A 16-year-old female presented to hospital, at which point tablets were found in her clothing. The clinicians could not ascertain what these tablets were, and wanted to establish whether they were relevant to her hospital presentation. Three distinct tablets were submitted for analysis (Figure 3.8).

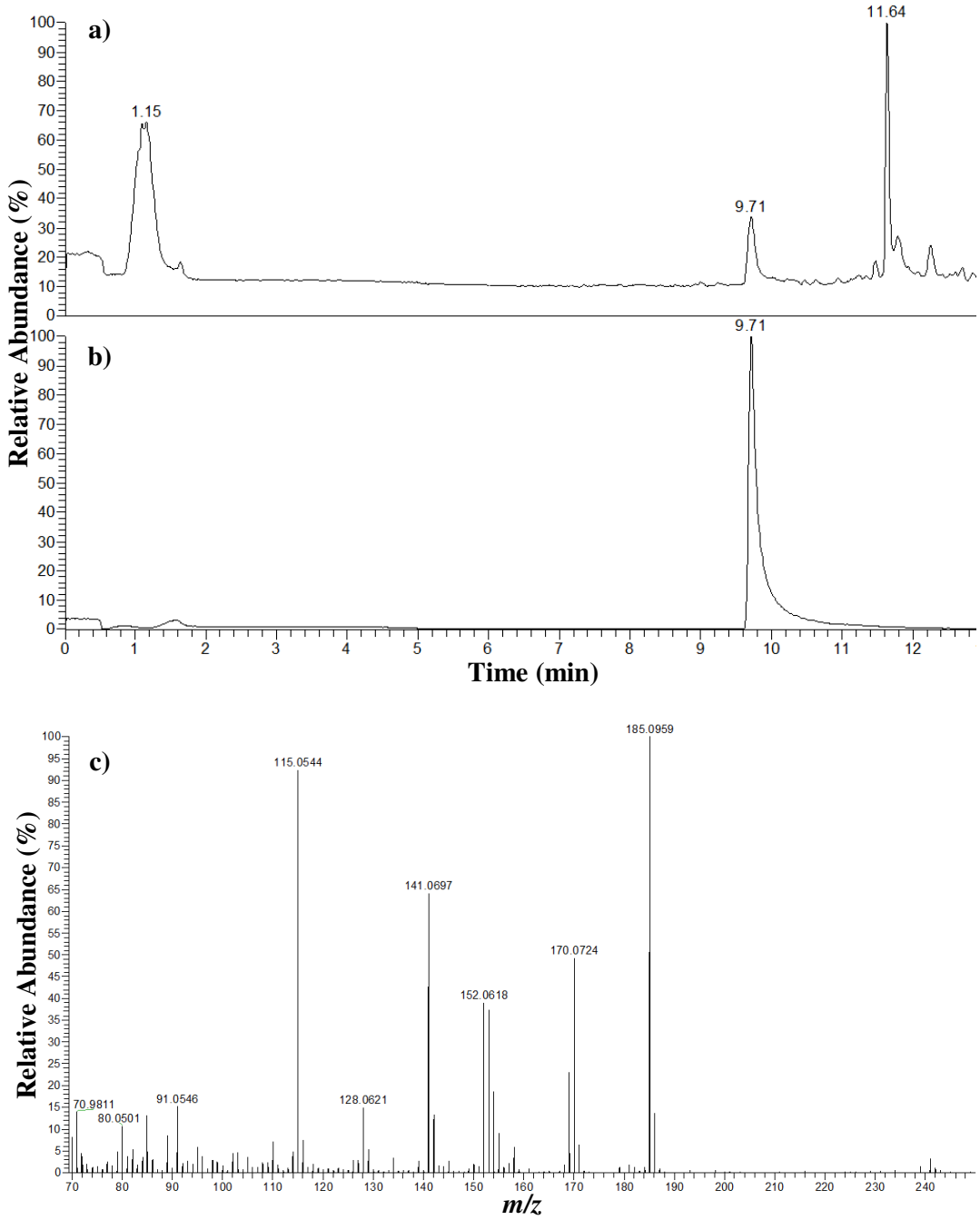
Figure 3.8 – Physical appearance and dimensions of the 3 unknown tablets

Tablet 1	Tablet 2	Tablet 3
		
11 mm diameter 3 mm thick	17 x 8 mm 5 mm thick	6 mm diameter 2 mm thick

3.3.2.1 Tablet 1

Analysis of the first tablet produced a TIC with a distinct peak at 9.71 min, which had a measured *m/z* of 231.1012 (Figure 3.9). From this, potential molecular formulae were generated, with the best match being $C_{14}H_{15}O_3$. A ChemSpider search of the formula yielded 1,351 results, with naproxen listed as the first match. The product ions for naproxen were ascertained as 185.0961, 170.0726, 153.0670, and 115.0542 from *mz*Cloud. All of these ions were identified in the MS^2 spectra at 9.71 min (Figure 3.9c). To confirm the retention time, an aqueous solution (1 mg/L) of a reference standard of naproxen (Sigma Aldrich) was analysed, and confirmed elution at 9.71 min.

Figure 3.9 – Analysis of an extract of Tablet 1 (see Figure 3.8) showing a) the TIC, b) an XIC for *m/z* 231.1012, and c) the MS^2 spectra at 9.71 min



3.3.2.2 Tablet 2

The second tablet had lettering on it, and was identified as paracetamol through searching the TICTAC database (Figure 3.10). On analysis, the TIC had a large peak at 3.57 min, with observed *m/z* 152.0703 (Figure 3.11). The theoretical *m/z* for paracetamol is 152.0706, giving a mass measurement error of -1.97 ppm.

The dominant product ion for paracetamol is *m/z* 110.0600 (*mz*Cloud). Other product ions include *m/z* 111.0441, 93.0335, and 65.0386. All product ions, with the exception of the 65.0386 ion, were detected in the MS^2 spectrum at 3.57 min. The 65.0386 ion was not detected as the lower limit for the scan range is set at 70 *m/z* in the method (Section 2.2.4).

Figure 3.10 – TICTAC record for Alvedon tablet

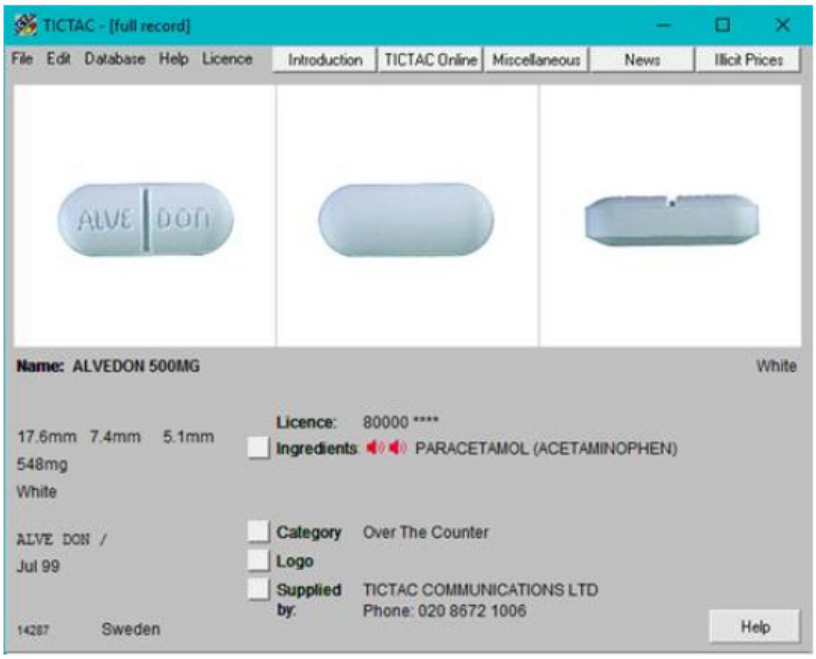
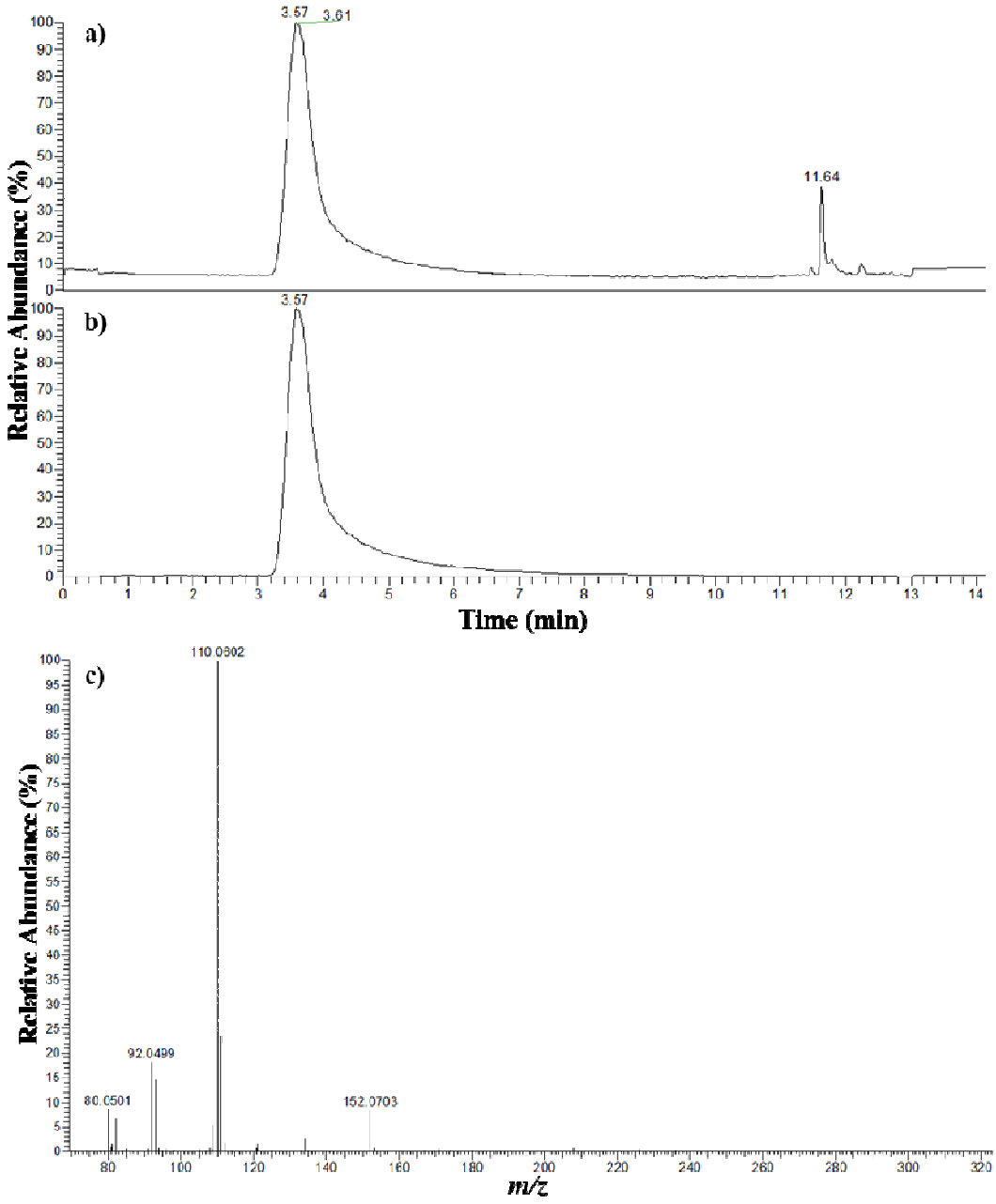


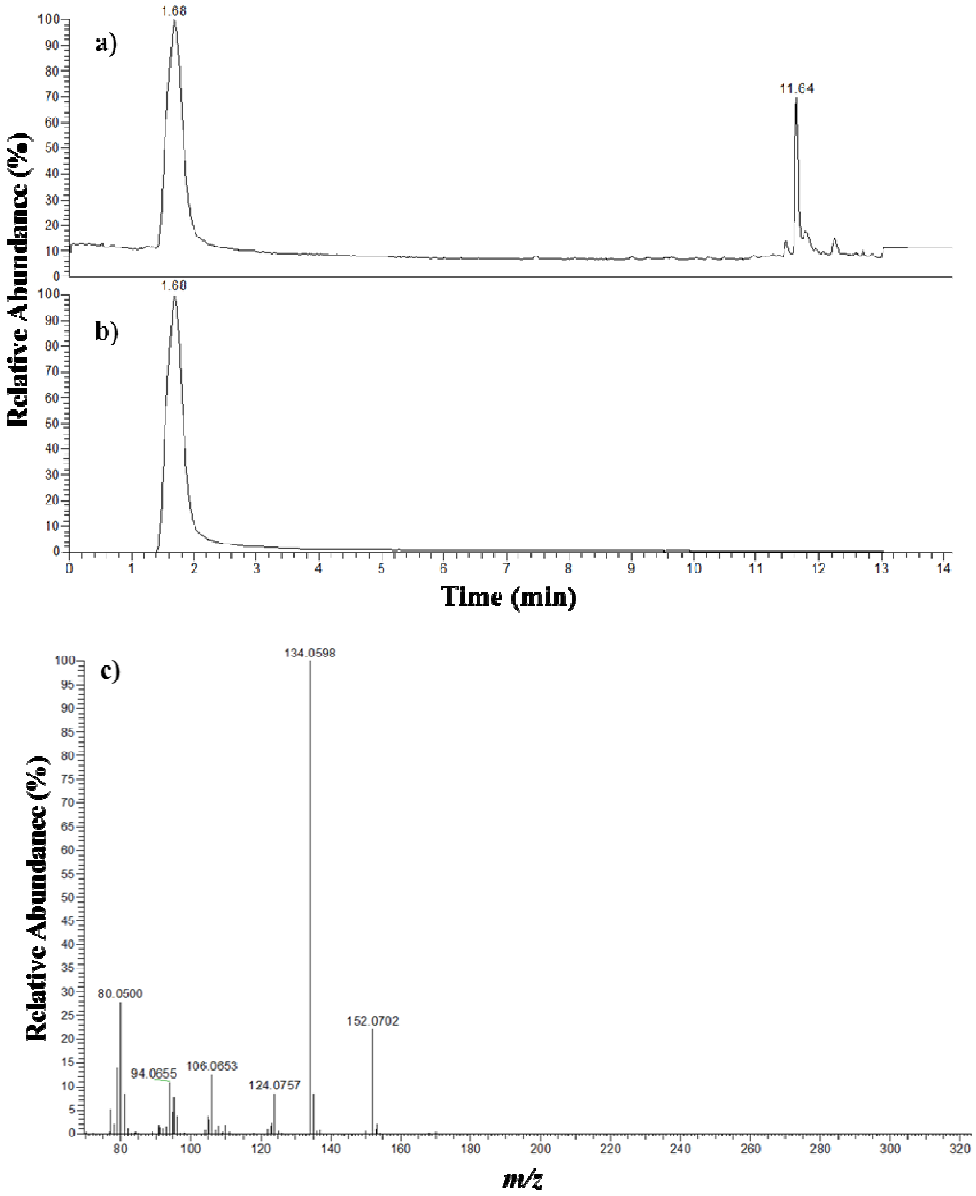
Figure 3.11 – Analysis of an extract of Tablet 2 (see Figure 3.8) showing a) the TIC, b) an XIC for paracetamol (*m/z* 152.0706), and c) the MS^2 spectra at 3.57 min



3.3.2.3 Tablet 3

Analysis of the third tablet produced a TIC with a large peak present at 1.68 min, with an observed *m/z* of 170.0808 (Figure 3.12). Potential molecular formulae were generated, with the best match being $C_8H_{12}O_3N$. A ChemSpider search of the formula yielded 1,290 results with pyridoxine listed as the first match. Pyridoxine (Vitamin B6) is a common dietary supplement. The main product ions for pyridoxine were obtained from *mz*Cloud as *m/z* 152.0706, 134.0600, and 124.0757, which were all present in the MS^2 spectrum at 1.68 min (Figure 3.12c).

Figure 3.12 – Analysis of an extract of Tablet 3 (see Figure 3.8) showing a) the TIC, b) an XIC for *m/z* 170.0808, and c) the MS^2 spectra at 1.68 min identifying product ions



3.3.2.4 Patient Urine Sample

To assess whether the patient had taken paracetamol and/or naproxen, a urine sample from the time when she was admitted to hospital was analysed. Vitamin B6 is naturally present in the body from dietary sources, thus additional intake cannot be readily assessed.

Only 2 % of a paracetamol dose is excreted as unchanged drug in urine, with the remainder excreted as conjugates; 45-55 % as a glucuronide, 20-30 % as a sulfate, and 15-55 % as cysteine and mercapturic acid conjugates (Baselt, 2014). A small peak for paracetamol was detected in the urine sample, and peaks corresponding to the masses of the sulfate and glucuronide metabolites were also apparent (Figure 3.13).

Naproxen is metabolised to *O*-desmethylnaproxen, with both compounds also undergoing glucuronidation. After a daily dose, approximately 10 % is excreted as unchanged drug, 60 % as naproxen glucuronide, 5 % as *O*-desmethylnaproxen, and 23 % as *O*-desmethylnaproxen glucuronide (Baselt, 2014). Naproxen was not detected in the urine sample. Possible peaks corresponding to the glucuronide metabolites were detected but could not be confirmed due to a lack of reference material (Figure 3.14).

Figure 3.13 – Extracted ion chromatograms for a) paracetamol (*m/z* 152.0706), b) paracetamol sulfate (*m/z* 232.0274), and c) paracetamol glucuronide (*m/z* 328.1027) in the patient's urine sample

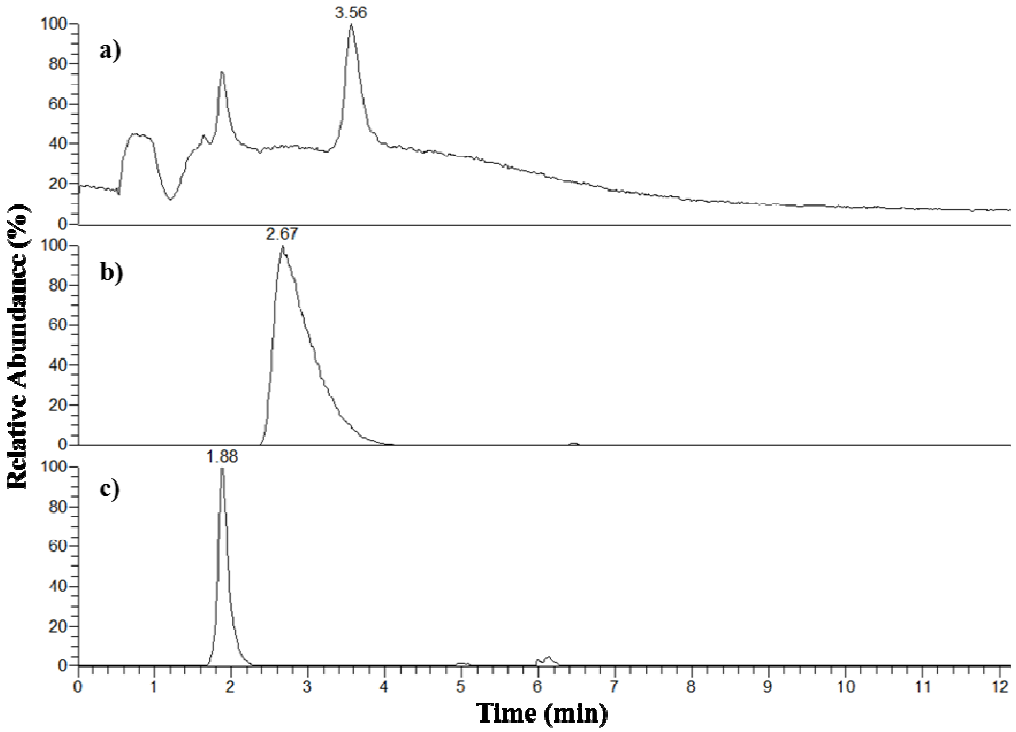
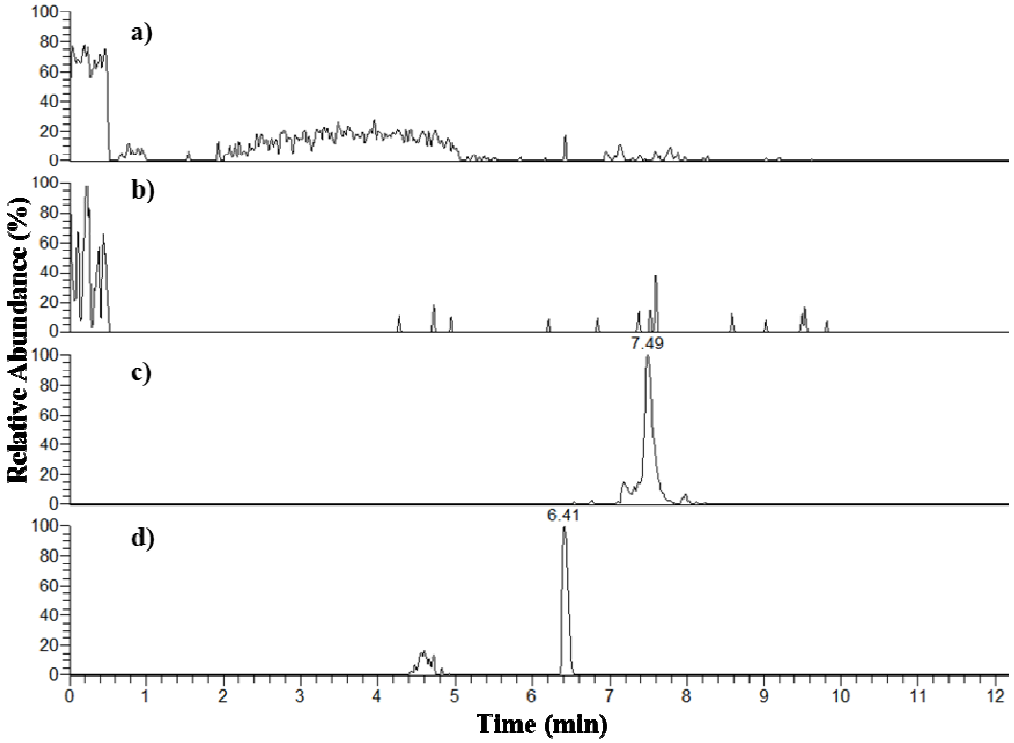


Figure 3.14 – Extracted ion chromatograms for a) naproxen (*m/z* 231.1016), b) *O*-desmethylnaproxen (*m/z* 217.0859), c) naproxen glucuronide (*m/z* 407.1337), and d) *O*-desmethylnaproxen glucuronide (*m/z* 393.1180) in the patient's urine sample



3.3.3 Possible use of a novel psychoactive drug

A urine sample from a 47-year-old male with a history of paranoid schizophrenia, anti-social personality disorder and polysubstance misuse (mainly stimulants) was submitted for screening. The individual was an inpatient at a psychiatric unit, but had unescorted leave for community outreach programmes. The clinician suspected he was obtaining and using a 'novel stimulant' whilst on leave, however the local biochemistry laboratory had reported negative results on their standard drug screen. At the time of sampling, the individual was prescribed olanzapine and venlafaxine.

The TIC showed many chromatographic peaks in the sample extract when compared to the negative urine calibrator (Figure 3.15), with most eluting between 6.5-8.5 min. The mass spectra for this time period showed four main ions; *m/z* 220.1315, 248.1624, 264.1936, and 278.2091 (Figure 3.16). These masses were extracted from the TIC to produce four separate XIC, which gave distinct chromatographic peaks (Figure 3.17). Knowing the individual was prescribed olanzapine and venlafaxine enabled expected masses to be screened for (Table 3.2). The two chromatographic peaks with *m/z* 264.1952 were identified as *N*- and *O*-desmethylvenlafaxine, and the single peak with *m/z* 278.2091 as venlafaxine, all by comparison with reference standards. The peaks with *m/z* 220.1327 and 248.1640 were not accounted for by prescribed medication and thus potential chemical structures were calculated in Xcalibur and subsequently searched in ChemSpider to identify potential compounds. ChemSpider gave 15,198 matches for $C_{15}H_{21}NO_2$ and 9,696 matches for $C_{13}H_{17}NO_2$. Recognisable drugs, with misuse potential, from these matches were further investigated (Table 3.3).

Individual aqueous solutions (1 mg/L) of reference standards for pethidine, methoxetamine and ritalinic acid (Sigma Aldrich) were analysed to ascertain the retention time for each compound. Neither pethidine, nor methoxetamine co-eluted with the peak observed for *m/z* 248.1641 (Figure 3.18) eliminating these drug from the investigation. The retention time for ritalinic acid matched the retention of the unknown compound of *m/z* 220.1329, and was further confirmed through identification of the product ion *m/z* 84.0808. Ethylphenidate was also confirmed in the sample through comparison with a reference standard (obtained from TICTAC Communications Ltd) for retention time and MS^2 product ions (Figures 3.19 and 3.20). Methylphenidate (Ritalin) was not detected in the sample, and thus was unlikely to have been the drug taken. Internet searches revealed that ethylphenidate is sold as a drug in its own right, with its use first reported to the European Union by the UK in 2011 (ACMD, 2015).

Figure 3.15 – Comparison of TIC of the patient urine sample (black) and negative urine calibrator (red) (see Section 3.3.3)

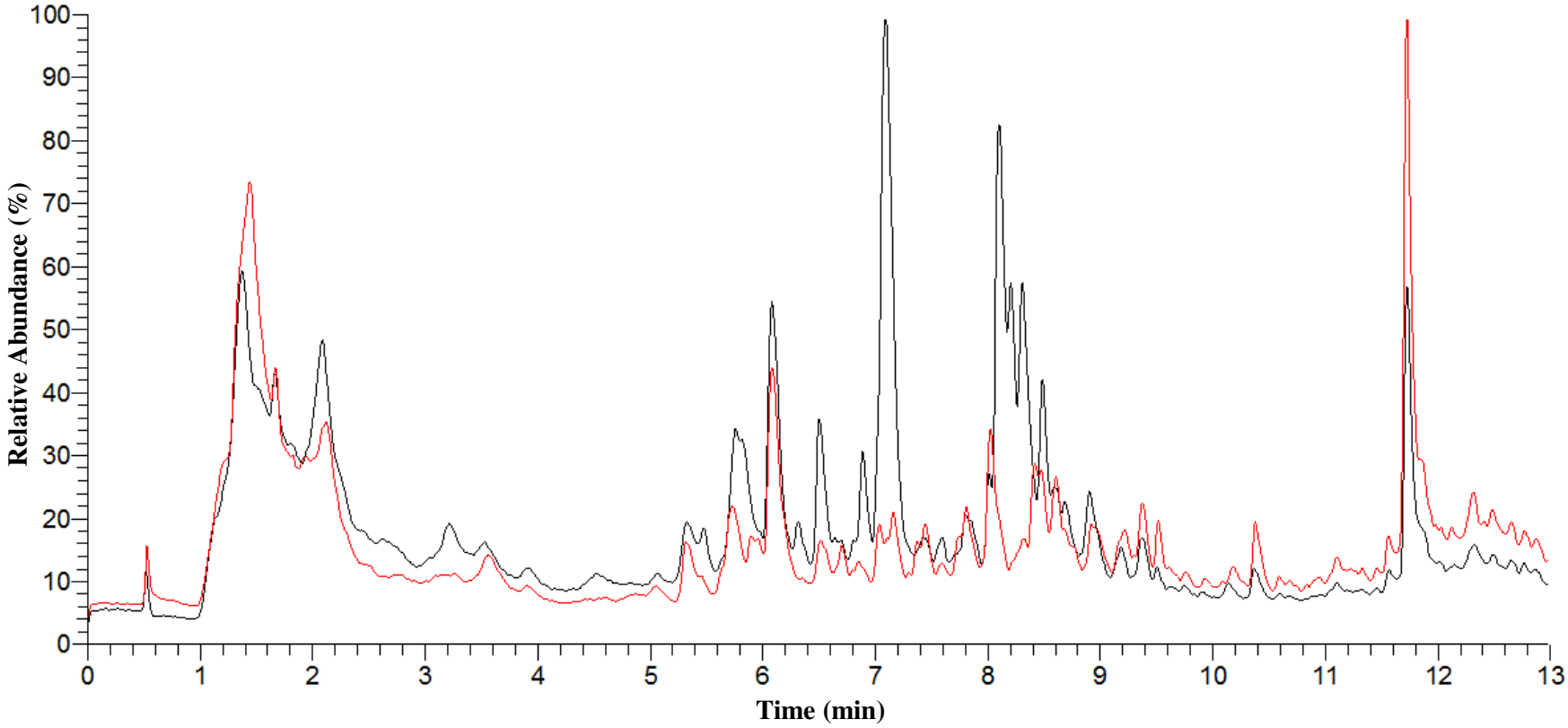


Figure 3.16 – Mass spectra from 6.5-8.5 min to identify the dominant precursor ions present in the sample (see Figure 3.15)

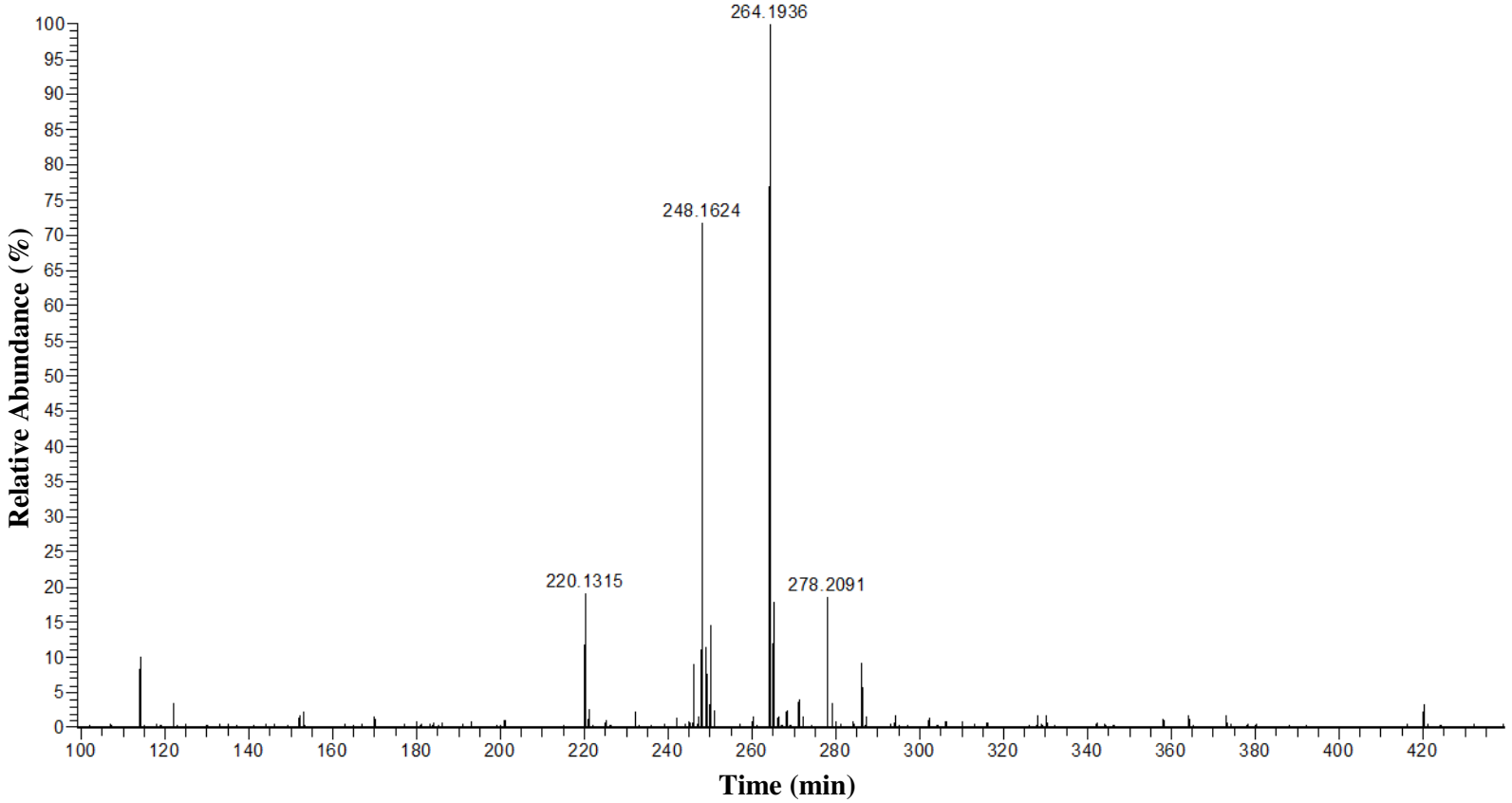


Figure 3.17 – Extracted ion chromatograms of the urine sample for the dominant precursor ions (see Figure 3.16): a) *m/z* 220.1327, b) *m/z* 248.1640, c) *m/z* 264.1952, and d) *m/z* 278.2091

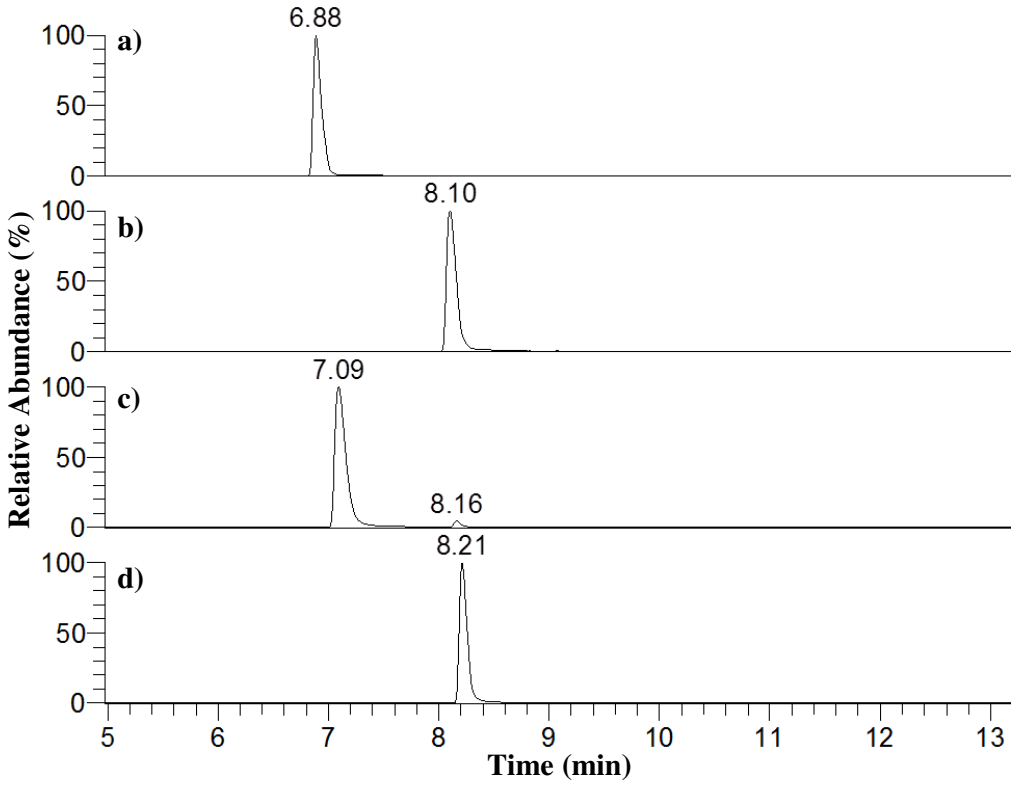


Table 3.2 – Expected *m/z* from venlafaxine, olanzapine and some urinary metabolites

Drug/metabolite	Expected *m/z*
Venlafaxine	278.2115
N- and *O*-Desmethylvenlafaxine	264.1958
N,O-Didesmethylvenlafaxine	250.1802
Olanzapine	313.1481
N-Desmethylolanzapine	299.1325

Table 3.3 – Potential compounds identified from ChemSpider searches which may have misuse potential

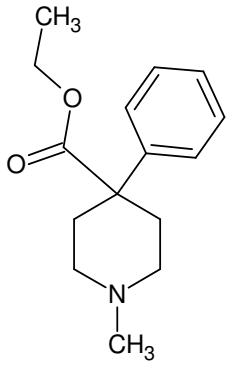
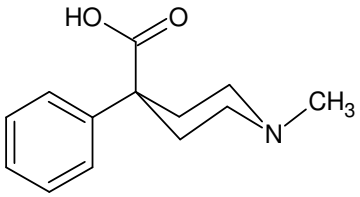
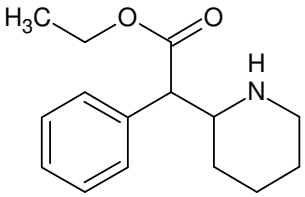
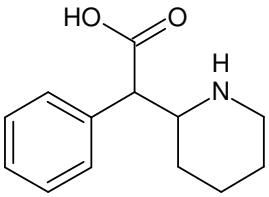
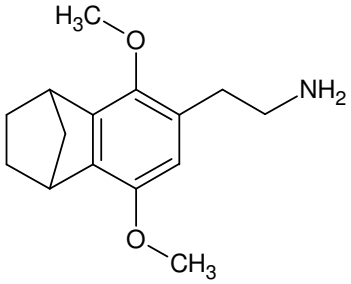
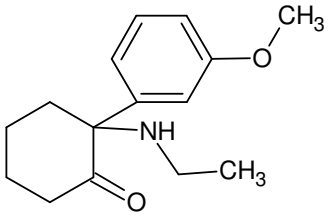
***m/z* 248.1641** **$C_{15}H_{21}NO_2$**	***m/z* 220.1329** **$C_{13}H_{17}NO_2$**
Pethidine 	Pethidinic acid 
Ethylphenidate 	Ritalinic acid 
2C-G-5 	2C-G-5 metabolite?
Methoxetamine 	Methoxetamine metabolite?

Figure 3.18 – Extracted ion chromatograms (*m/z* 248.1641) for a) the patient urine sample, b) methoxetamine reference standard, and c) pethidine reference standard.

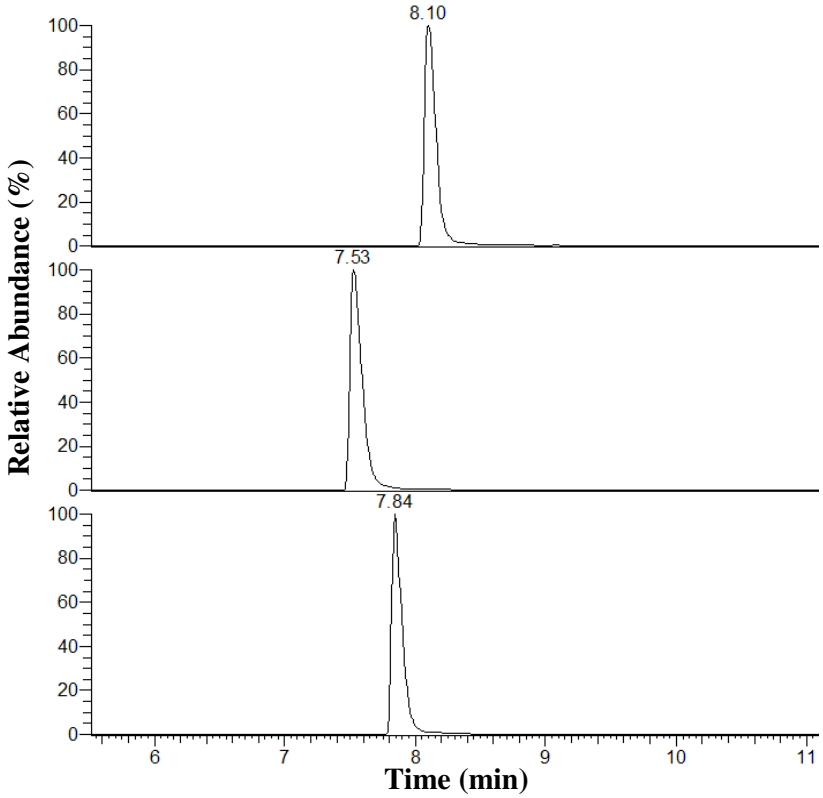


Figure 3.19 – Comparison of MS^2 spectra at 8.10 min for ethylphenidate and at 6.88 min for ritalinic acid in a) the patient sample, and b) reference standards

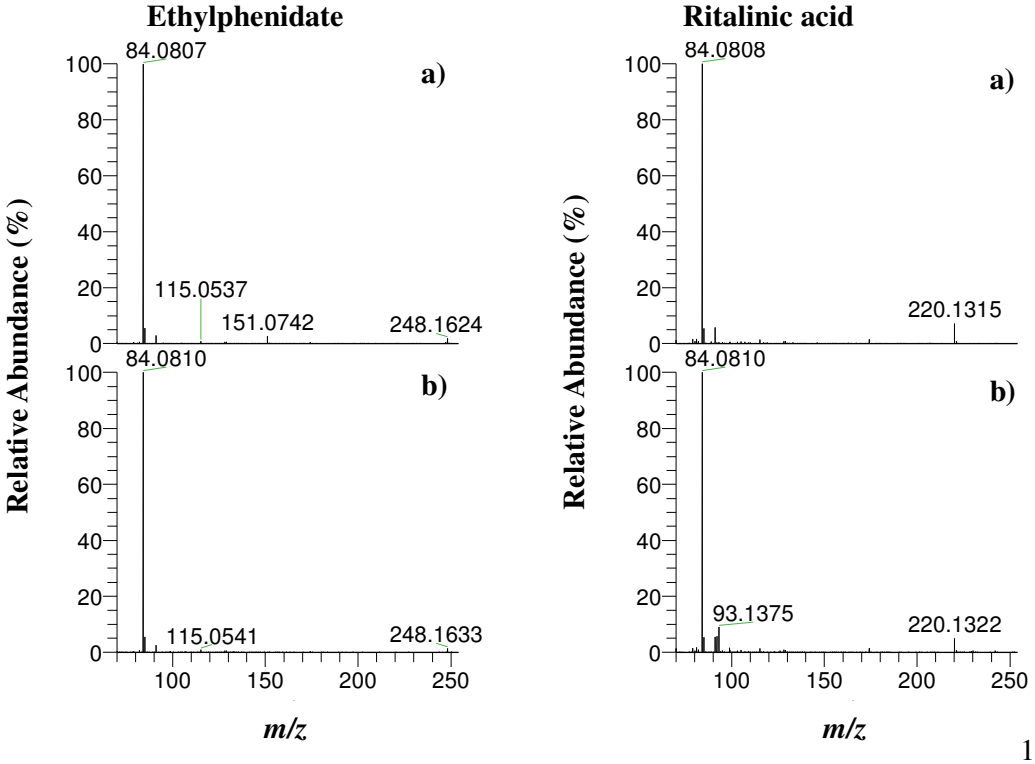
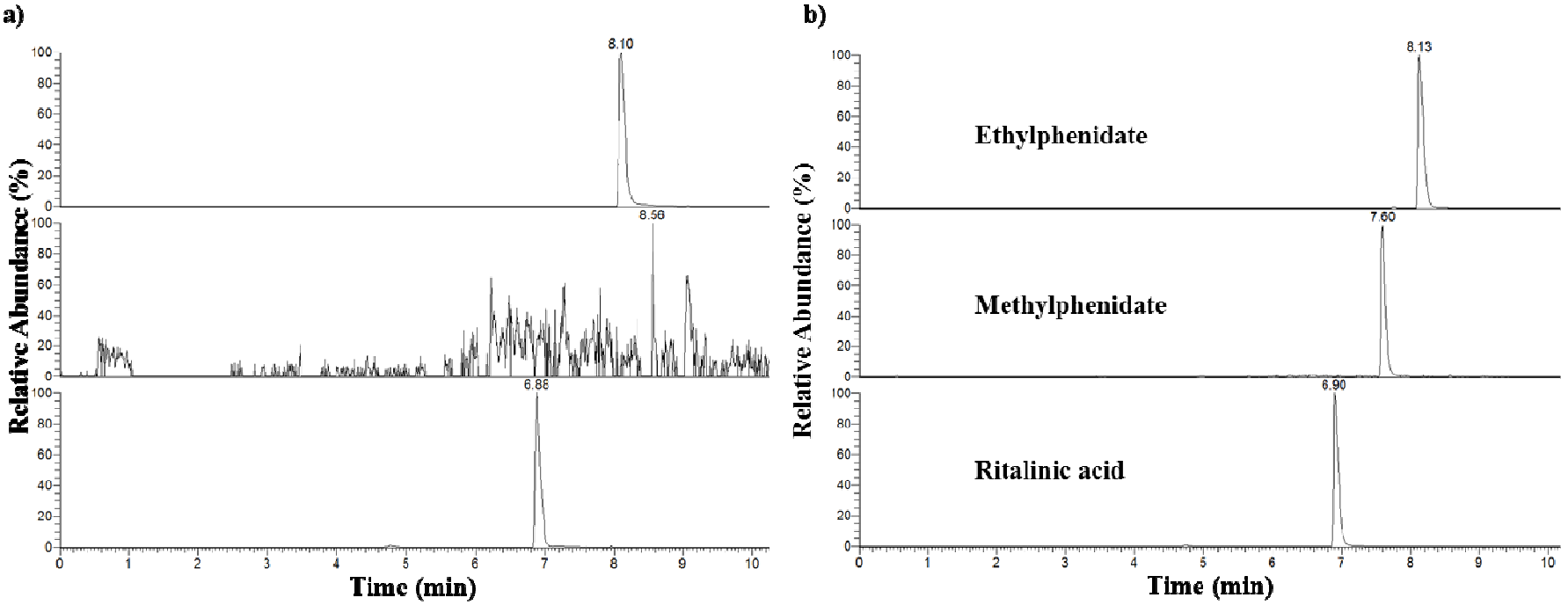


Figure 3.20 – Comparison of XIC for ethylphenidate (*m/z* 248.1641), methylphenidate (*m/z* 234.1489), and ritalinic acid (*m/z* 220.1332) in a) the patient sample, and b) reference standard solutions



3.3.4 Sedation of a young child

A 7-year-old child presented to an accident and emergency department floppy and unresponsive. He was stabilised and monitored, and his condition improved. His condition was noted to worsen again after his parents visited. At this time a packet of diazepam was found near the child's bed. Ward clinicians suspected that the child was being given the diazepam, which would account for his sedated state. However, immunoassay screening results done at a local biochemistry laboratory were negative for benzodiazepines. The sample was referred for analysis by LC-HRMS, but neither diazepam nor its metabolites were detected in the urine sample (Figure 3.21).

To ascertain if other compounds were present in the urine sample, the TIC for the sample was compared against that of the negative calibrator. This revealed peaks present in the urine sample between 5.3-5.7 min (Figure 3.22). The observed *m/z* from this time period showed the dominant ion to be *m/z* 405.1072 (Figure 3.23a). From studying the mass spectra, a chlorine atom was identified in the compound (presence of the M+2 peak; Figure 3.23b).

Potential molecular formulae were generated using Xcalibur (Table 3.4); from these one formula contained a single chlorine atom and showed the smallest deviation in mass to the observed *m/z*. ChemSpider search results for "$C_{17}H_{17}O_4N_6Cl$" gave 39 possible compounds. One of the compounds identified was zopiclone *N*-oxide.

Zopiclone is a 'Z-drug' that has similar pharmacological properties to benzodiazepines, thus ingestion of this drug was compatible with the clinical picture. In cases of overdose, an individual may present with excessive sedation and depressed respiration (Boniface and Russell, 1996). Zopiclone is extensively metabolised; the principal metabolites are *N*-desmethylzopiclone and zopiclone *N*-oxide (Figure 3.24). A single oral dose is excreted in the 24 h urine as parent drug (4.5 %), zopiclone *N*-oxide (11 %), and *N*-desmethylzopiclone (15 %) (Baselt, 2014).

The full scan data were filtered for zopiclone and both metabolites, with the retention time for zopiclone confirmed through comparison with an aqueous solution (1 mg/L) of reference standard (Sigma Aldrich) (Figure 3.25). Product ions for zopiclone were identified as 263.0330, 245.0225, 217.0276, and 130.0052 from *mz*Cloud, and were all present in the MS^2 spectrum at 5.14 min.

Figure 3.21 – Comparison of XIC filtered for diazepam and selected metabolites in a) the urine sample, and b) reference solutions (see Section 3.3.4)

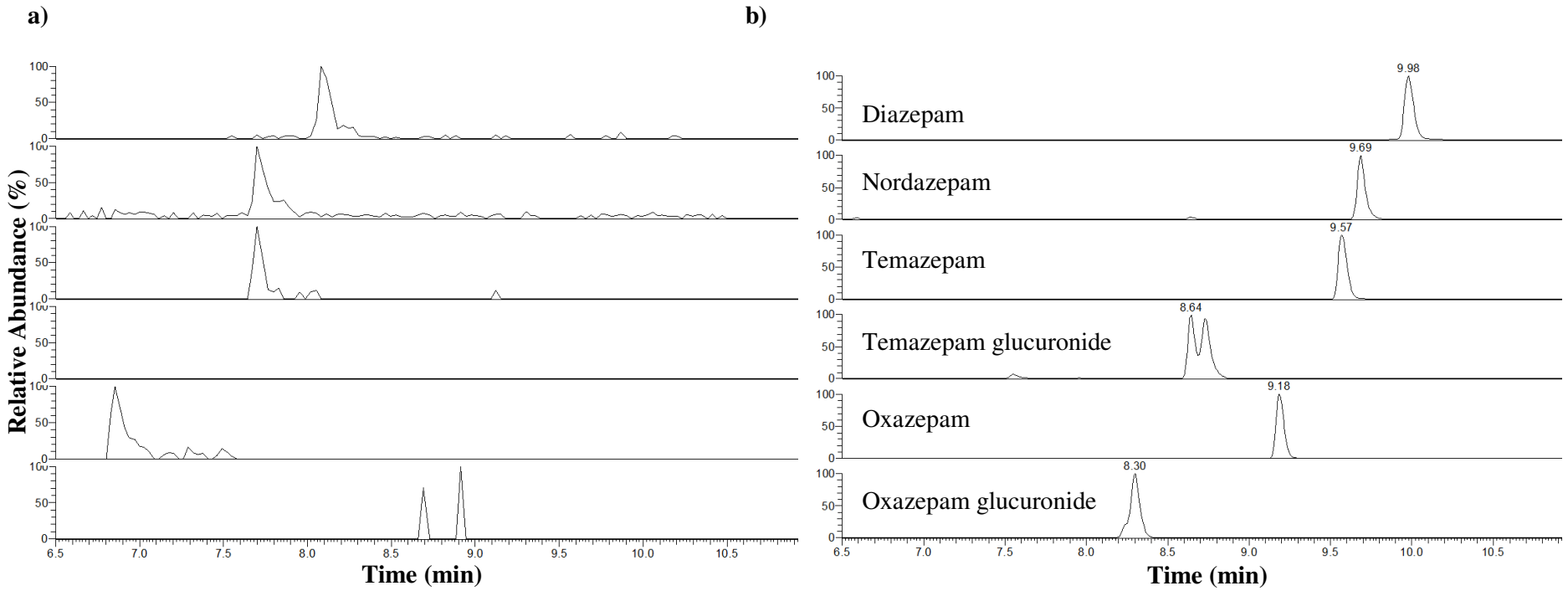


Figure 3.22 – Comparison of TIC of the urine sample (black) and negative urine calibrator (red)

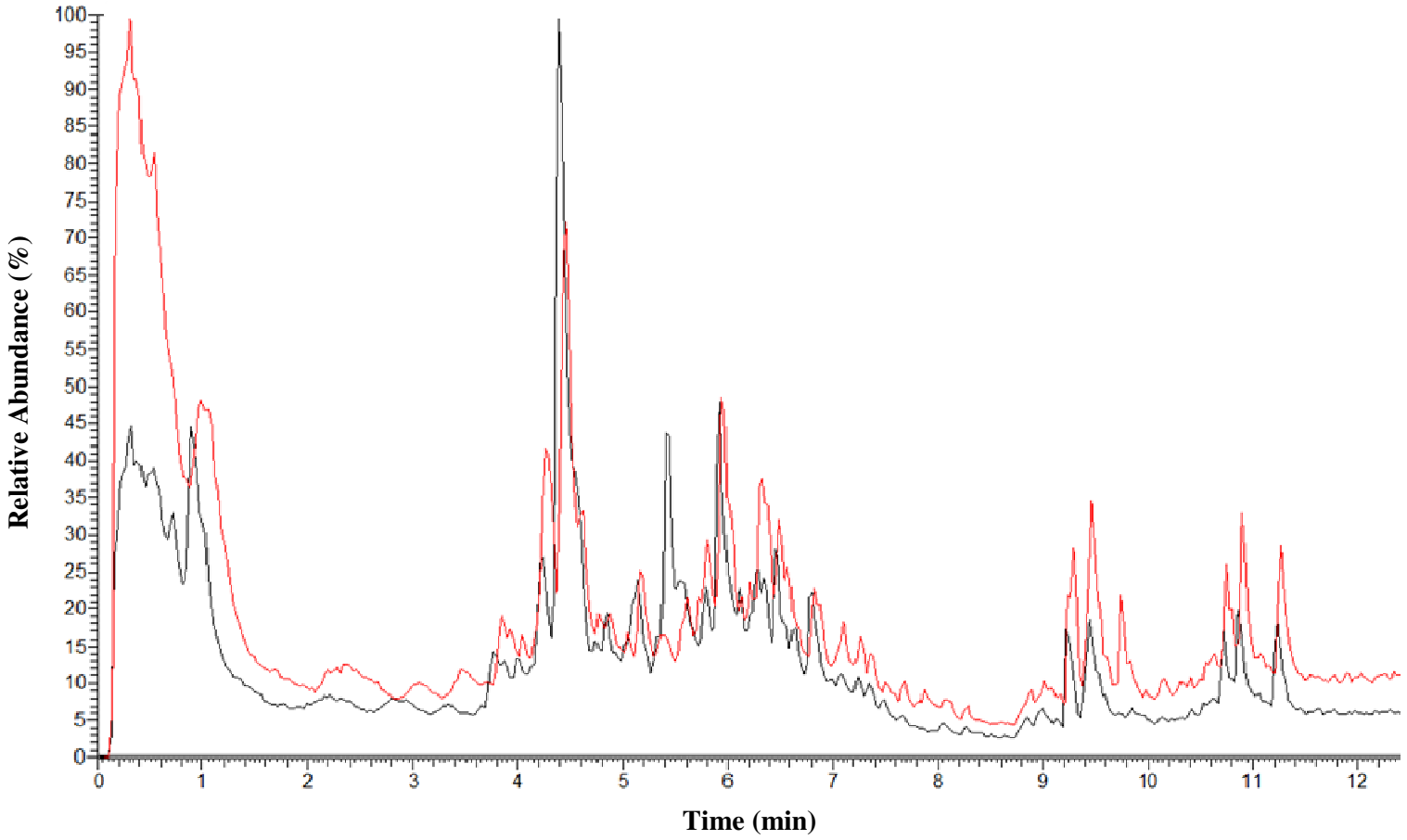


Figure 3.23 – Mass spectra from 5.3-5.7 min showing a) scan range *m/z* 100-750, and b) scan range *m/z* 400-415

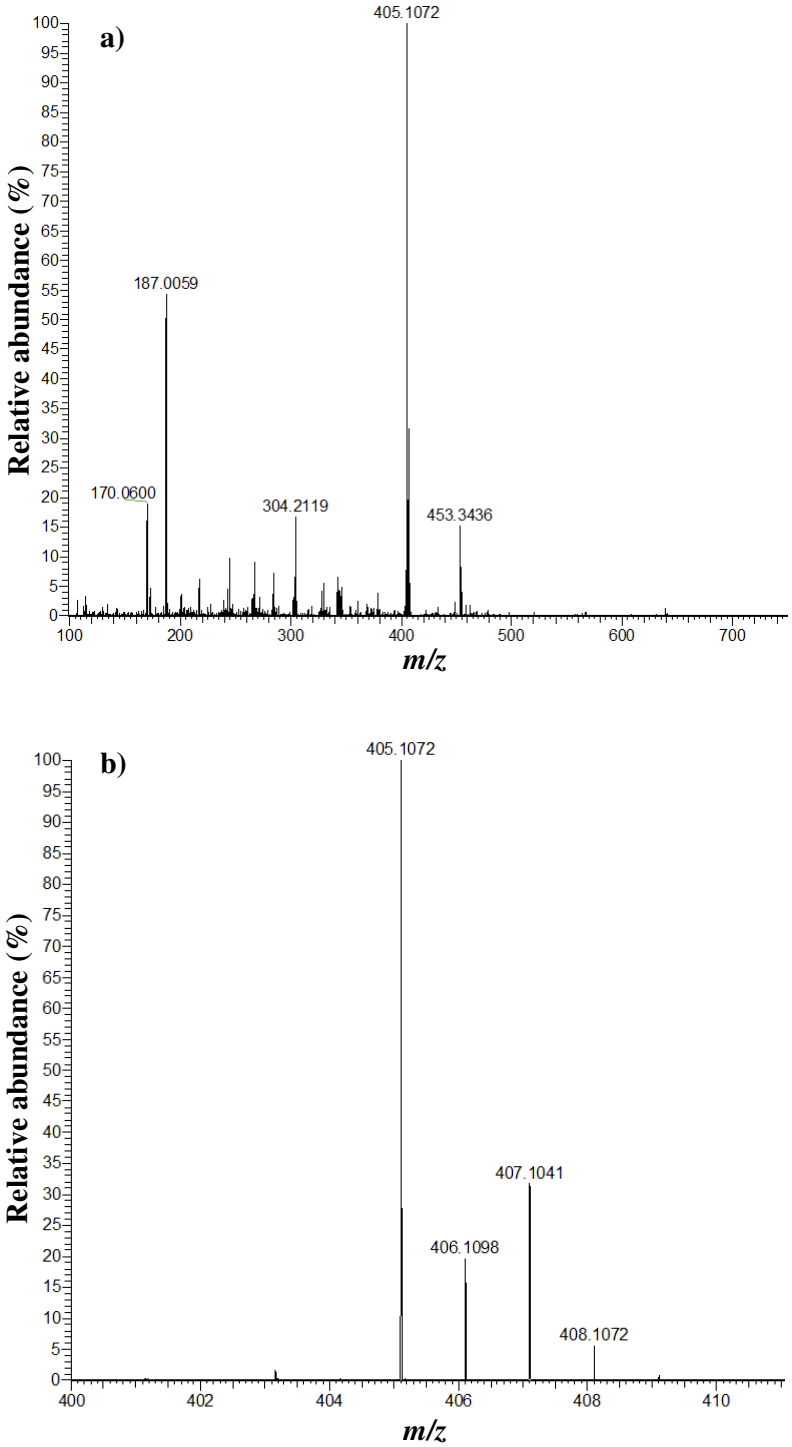


Table 3.4 – Top 5 elemental composition suggestions based on the observed *m/z* 389.1123 from Xcalibur software

	Molecular formula	Mass difference (mmu)
1	$C_{17}H_{18}O_4N_6Cl$	-0.03
2	$C_6H_{17}O_{11}N_{10}$	-0.05
3	$C_{20}H_{15}O_5N_5$	0.46
4	$C_{14}H_{21}O_3N_7Cl_2$	-0.51
5	$C_{15}H_{27}O_8Cl_2$	-0.52

Figure 3.24 – Metabolic pathway of zopiclone

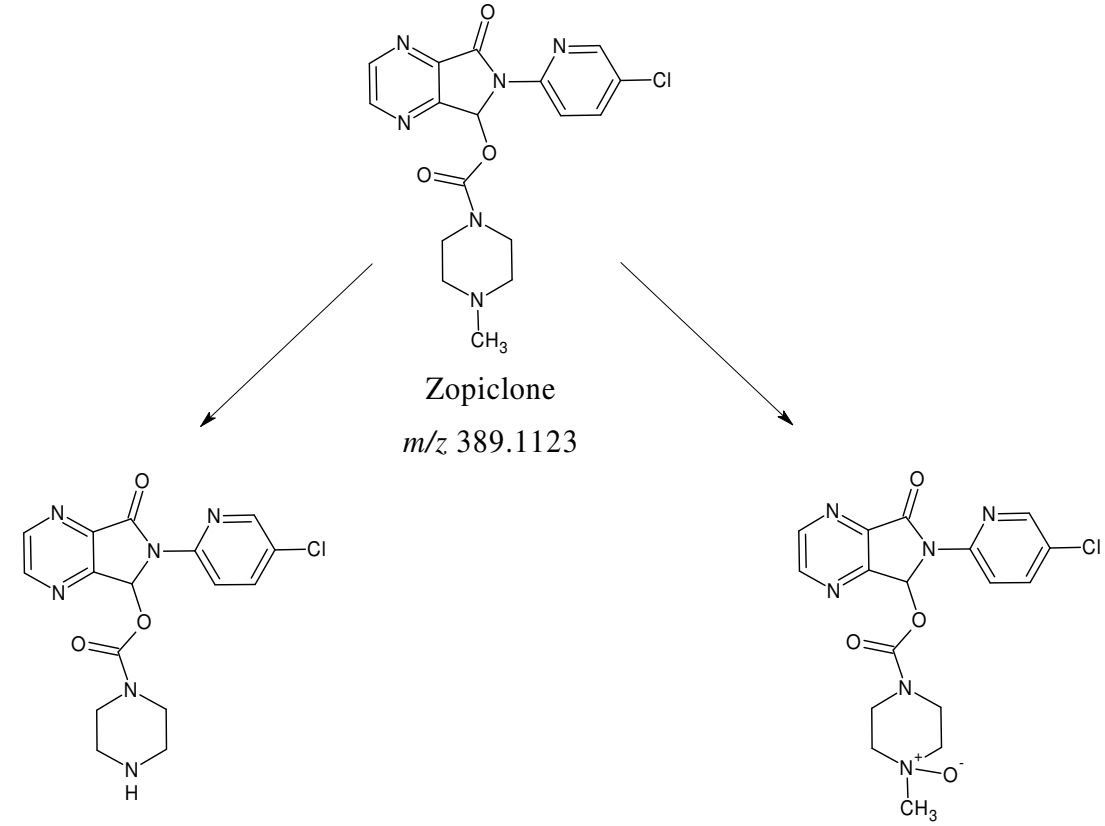
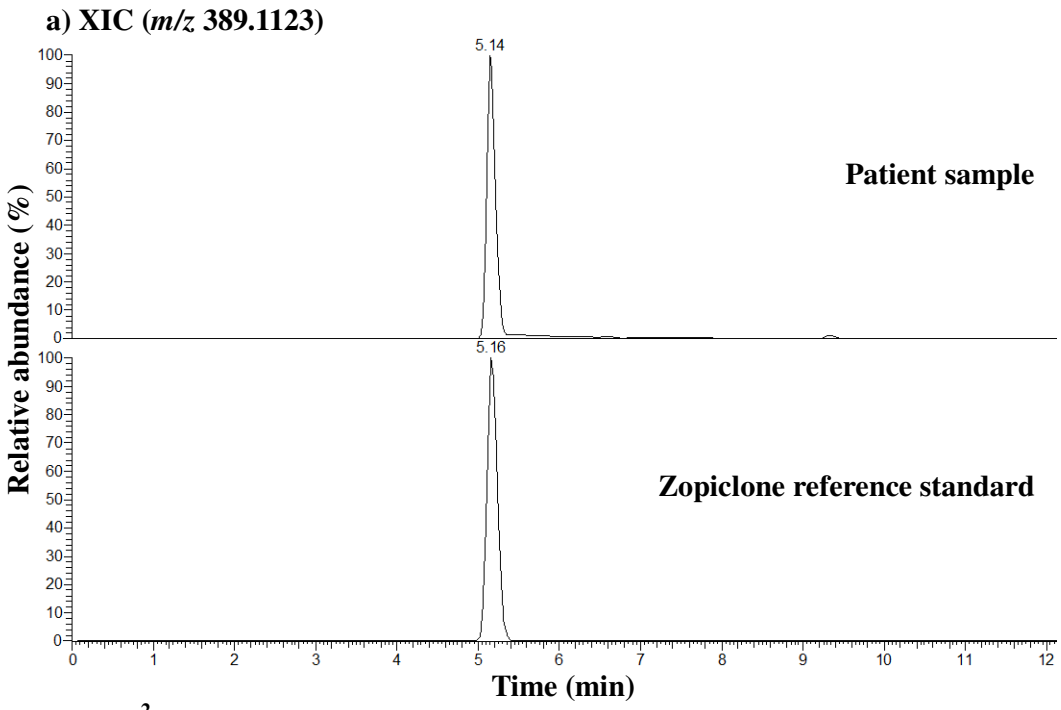


Figure 3.25 – Confirmation of zopiclone by a) retention time, b) product ions, c) isotope ratio, and d) presence of metabolites



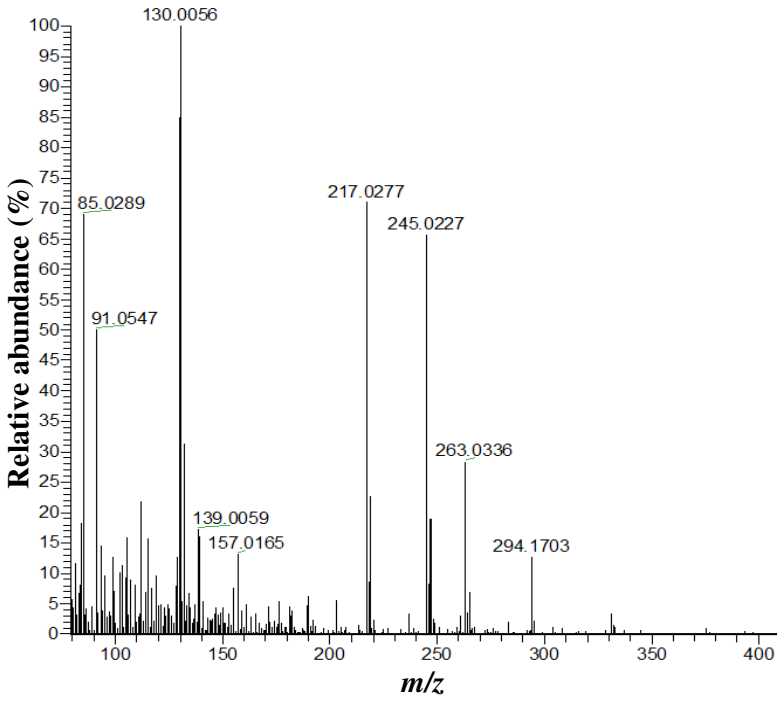
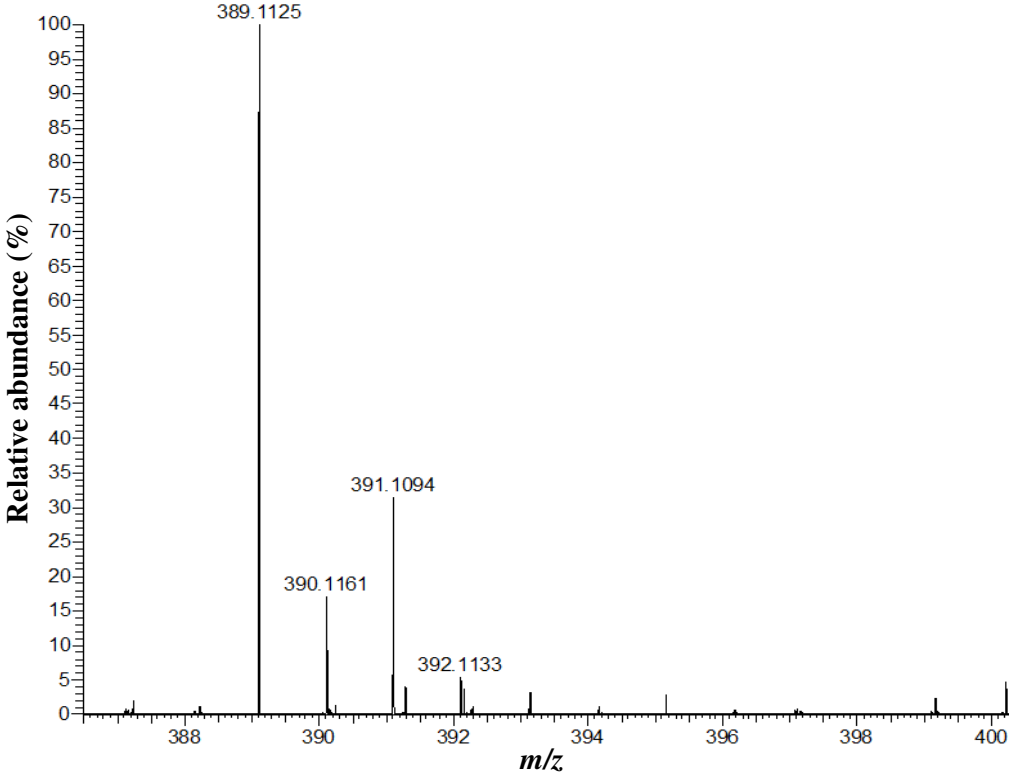
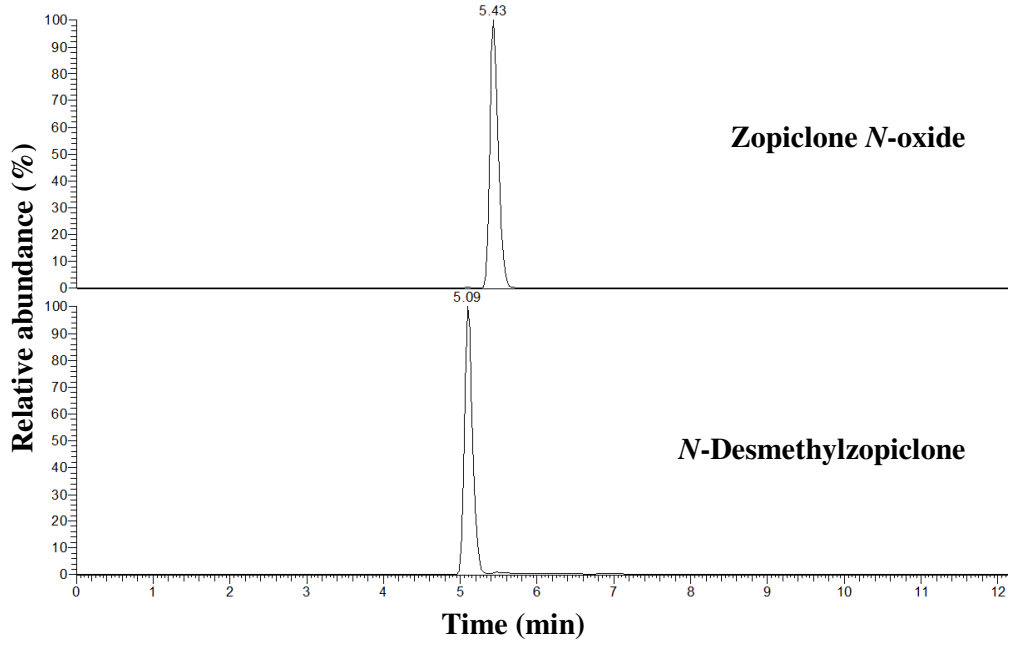


Figure 3.25 (cont.) – Confirmation of zopiclone by a) retention time, b) product ions, c) isotope ratio, and d) presence of metabolites

c) Full MS scan at 5.14 min confirming the presence of chlorine in the molecule



d) XIC for metabolites



3.4 Conclusions

LC-HRMS is a powerful analytical tool for aiding compound identification either in solid form, or present within a biological sample. However it is important to note that even high mass accuracy (<1 ppm) alone is not enough to exclude all potential candidate compounds. For definitive identification a reference standard of the compound is required to enable direct comparison of spectra and retention time. In some cases it may not be possible to identify compounds present in a substance, and some substances may not be soluble in methanol or may be at too low concentration to enable detection.

Screening for unknown compounds using LC-HRMS remains a largely manual process. It is therefore important to glean as much background information on the substance and patient history (including prescribed medications) from clinicians or others. Useful information may include clinical features, the origin of any substance they have taken (e.g. bought on a website), product name, and product claims (e.g. sexual performance, weight-loss aid). By gaining this information, initial investigations can be targeted towards potential drugs/drug classes.

3.4.1 Further work

Continual work in building up an 'in-house' database is required to aid compound identification. This task is on-going as drugs, particularly NPS, which are available on the street are constantly changing. Compilation of a laboratory and method specific database of pure reference compounds is of benefit to unknown screening using LC-HRMS, and may enable faster identification of an unknown compound. The database should include as many confirmation criteria as possible. Inclusion of retention time data (specific to a laboratory's method) is crucial to enable compound identification, and may enable differentiation of isobaric compounds.

As demonstrated, manual interrogation for unknown substances is a complex process which can be time-consuming and is dependent on the analyst's level of expertise. For unknown screening to become more commonplace in a clinical laboratory significant improvement in data processing software is required. As software is developed by instrument manufacturers, it will be important to test whether it is suitable for purpose and identify any pitfalls that arise in practice.

4 Incorporation of new analytes into an existing LC-HRMS method: Achiral analysis of methylphenidate, ethylphenidate, and ritalinic acid in human urine

4.1 Introduction

4.1.1 Methylphenidate

Methylphenidate is a psychostimulant prescribed as first-line treatment in Attention Deficit Hyperactivity Disorder (ADHD) (Seixas *et al.*, 2012). Methylphenidate hydrochloride is supplied as a racemic mixture, but its therapeutic effects are mainly due to the *R,R*- isomer (Heal and Pierce, 2006; Kimko *et al.*, 1999). Methylphenidate acts by blocking dopamine and noradrenaline transporters through allosteric binding, and thus inhibits catecholamine reuptake and increases catecholamine availability in the synaptic cleft in cortical and subcortical regions (Arnsten and Pliszka, 2011; Kuczenski and Segal, 2002; Seu *et al.*, 2009).

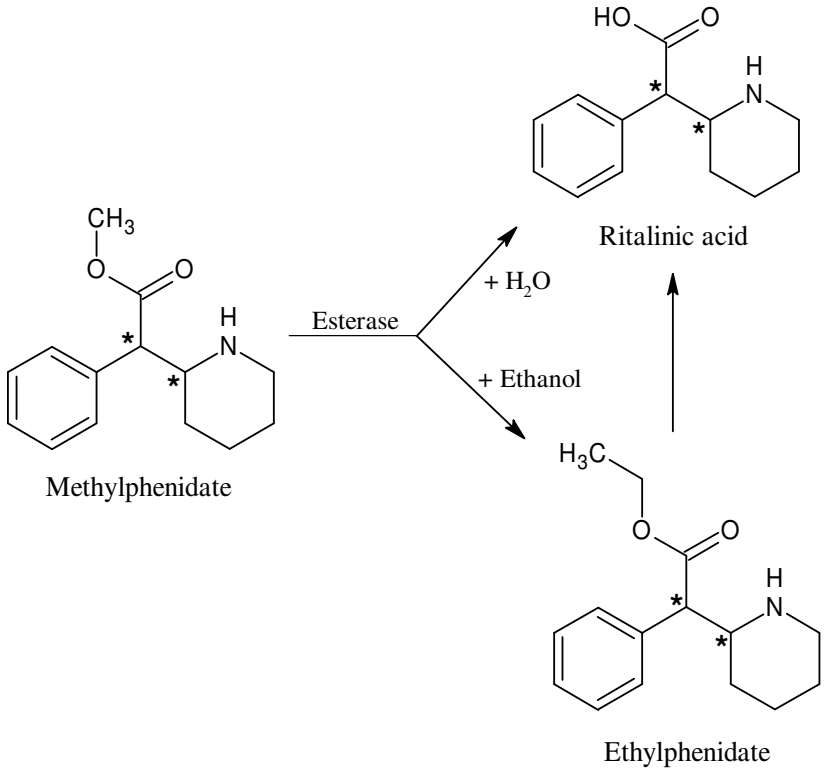
Methylphenidate is prescribed for oral use and is available in immediate (e.g. Ritalin, Novartis) or prolonged-release (e.g. Concerta XL, Janssen-Cilag) formulations. However, different routes of administration, such as insufflation or intravenous injection, may be used to increase bioavailability when methylphenidate is abused for its stimulant properties. After oral administration, methylphenidate is rapidly and completely absorbed, but is hydrolysed quickly to an inactive metabolite, ritalinic acid (Figure 4.1). Less than 1 % of an oral dose of methylphenidate is excreted unchanged in urine, whilst 80 % is excreted as ritalinic acid (Baselt, 2014). The peak duration of action is around 2-4 h for immediate release, and 3-8 h for sustained release formulations. When methylphenidate is taken concurrently with ethanol, *S,S*-ethylphenidate may be formed by enantioselective transesterification (Dinis-Oliveira, 2017; Patrick *et al.*, 2013). More recently, ethylphenidate has been marketed as a 'legal high' (Ho *et al.*, 2015; Krueger *et al.*, 2014). Ethylphenidate is also metabolised to ritalinic acid. Details of six other phenidate analogues marketed as 'legal highs' have been published (Klare *et al.*, 2017; Markowitz *et al.*, 2013). Of these, the alkyl analogue isopropylphenidate, and possibly also *N*-benzyl-ethylphenidate, would be likely to give rise to ritalinic acid *in vivo*.

Marked inter-individual variability in methylphenidate dose-response is recognised, and thus the dose is titrated to gain optimal effect and minimise the risk of toxic effects such as hypertension (Castells *et al.*, 2011; Faraone *et al.*, 2004; Scharman *et al.*, 2007). Adherence to treatment is also an important factor that may influence outcome (Retz *et al.*, 2012), and therapeutic drug monitoring would be of particular relevance given the serious consequences of inadequate treatment (Kessler *et al.*, 2006; Pitts *et al.*, 2015). Although metabolism is enantioselective after oral dosage, the plasma concentrations of

R,R-methylphenidate being higher than those of *S,S*-methylphenidate, the plasma half-life of total methylphenidate is very short (1-4 h) and degradation to ritalinic acid continues in plasma *in vitro*, limiting the value of plasma methylphenidate assay. However, methylphenidate and ritalinic acid are stable in urine for at least 6 months when stored at -20 ^{o}C (Paterson *et al.*, 2012). No formal stability studies have been conducted for ethylphenidate in urine, but it would be expected to behave in a similar manner to methylphenidate.

Figure 4.1 – Metabolic pathways of methylphenidate and ethylphenidate

* chiral centre



4.1.2 Aims

This chapter aims to explore one of the benefits of LC-HRMS, i.e. rapid incorporation of new analytes into existing analytical methods. Three analytes (methylphenidate, ethylphenidate and ritalinic acid) will be added to the LC-HRMS method for drug detection in urine (Chapter 2). Measurement of methylphenidate and metabolites may aid clinicians to monitor adherence to methylphenidate treatment and also to detect illicit use of either methylphenidate, or ethylphenidate. In order to explore the utility of the assay for assessing patient adherence, methylphenidate, ethylphenidate and ritalinic acid will be measured in urine samples from a small cohort of adult ADHD patients. Whether ingestion of ethylphenidate can be differentiated from concurrent use of methylphenidate and ethanol will also be explored.

4.2 Materials and Methods

4.2.1 Chemicals and Reagents

Methanolic solutions of racemic methylphenidate, ethylphenidate, methylphenidate-D_9 and ritalinic acid-D_{10}, and solid ritalinic acid (racemic mixture) were from Sigma Aldrich (Poole, UK). Screw-cap polypropylene tubes (2 mL) were from Alpha Laboratories (Eastleigh, UK). For all other chemicals and reagents see Section 2.2.1.

4.2.2 Instrumentation

All instrumentation is detailed in Section 2.2.2.

4.2.3 Preparation of Calibration and Internal Quality Control Solutions

Individual stock solutions of methylphenidate and ethylphenidate (1000 mg/L) were used as supplied, and a stock solution of ritalinic acid (100 mg/L) was prepared in methanol. Stock solutions were diluted as appropriate with methanol to give a working solution (10 mg/L each methylphenidate, ethylphenidate and ritalinic acid). All solutions were stored at -20 °C when not in use. Appropriate volumes of either the working solution or stock solutions were evaporated to dryness and reconstituted with analyte-free human urine to prepare the calibrators (0.05, 0.50, 5.00 mg/L) and IQC solutions (0.10, 0.25, 0.75 and 2.50 mg/L). Calibrator and IQC solutions were stored in approximately 500 µL portions in 2 mL screw-cap polypropylene tubes at -20 °C until needed.

4.2.4 Internal Standard Solution

A working IS solution containing methylphenidate-D_9 and ritalinic acid-D_{10} (both 0.05 mg/L) was prepared by appropriate dilution of individual stock solutions (both 100 mg/L) with eluent A (see Section 2.2.3).

4.2.5 Sample Preparation

Centrifuged urine samples/calibrators/IQCs (50 µL) were diluted with 450 µL working IS solution using an automated Hamilton dilutor directly into HPLC vials. The vials were capped and transferred to a pre-cooled (10 ^{o}C) autosampler tray.

4.2.6 Liquid Chromatography and Mass Spectrometry

The LC and MS method settings were identical to those used for the LC-HRMS method described in Chapter 2 (Sections 2.2.3 and 2.2.4).

4.2.7 Assay Calibration and Acceptance Criteria

Calibration standards (N = 3) and a matrix blank (analyte-free urine) were included at the beginning and end of each batch analysis, with IQCs included after the first set of calibrators and immediately before the last set of calibrators. Alternating IQC solutions were analysed after every 10 patient samples throughout the batch. Assay acceptance criteria were (i) linear (R^2 >0.98) calibration curves for each analyte, and (ii) at least 67 % (4 out of 6) of IQC samples within 20 % of their respective nominal value.

Peak area ratios (methylphenidate and ethylphenidate to methylphenidate-D_9; ritalinic acid to ritalinic acid-D_{10}) obtained on analysis of the calibration standards were plotted against concentration to construct calibration graphs. Linear regression intercepts were not forced through zero, and no line weighting was applied. Samples with analyte concentration >10 mg/L were diluted with analyte-free urine to within the calibration range and re-analysed.

4.2.8 Method Validation Protocol

Intra- and inter-assay accuracy (% nominal concentration) and precision (% RSD) was assessed for each analyte through replicate analysis of IQC solutions (N = 5) on the same day, and singleton analysis on 5 different days, respectively. The LoD was ascertained through successive serial dilution (1+1, v/v with analyte-free urine) of the low calibrator solution (0.05 mg/L) and was based upon the concentration at which the signal-to-noise ratio was >3, and the % RSD <20 (N = 5). The LLoQ was ascertained through assessing accuracy and precision of the low calibrator solution (0.05 mg/L). Linearity was assessed by replicate analysis (N = 6) of each calibrator, and was based upon accuracy within 80-120 % of the recalculated concentration. To assess whether sample dilution to within the calibration range was suitable, a 10 mg/L solution (containing all analytes) was diluted using analyte-free urine to within the calibration range, and was based upon accuracy within 80-120 % and the % RSD <20 (N = 3). To investigate matrix effects, solutions containing all analytes and internal standards were prepared in (i) deionised water, and (ii) analyte-free human urine from 10 independent sources. Prepared solutions were diluted (1+9, v/v) with eluent A and analysed in duplicate. The ratio of the peak area for each analyte to that of the relevant internal standard in analyte-free human urine samples was compared to that in deionised water. Carryover was assessed through consecutive analysis of a urine sample containing low (L) and high (H) analyte concentrations in the order L,L,L,H,H,H,L,L,L (L: all analytes 0.05 mg/L, H: all analytes 10 mg/L). Finally, a urine sample from a 47-year-old male

suspected of ingesting ethylphenidate submitted for screening for the presence of drugs of abuse by LC-HRMS (Section 3.3.3) was analysed to assess the capability of the method in distinguishing use of ethylphenidate from use of methylphenidate.

4.2.9 Patient Samples

Anonymised random urine samples were collected into sterile plastic containers from 27 adult male patients, who were diagnosed with ADHD according to the Diagnostic and Statistical Manual of mental disorders, and prescribed Concerta XL [N = 26, mean (range) daily dose 54 (18-90) mg/d] or Ritalin [N = 1, daily dose 20 mg]. Samples were stored at -20 ^{o}C for no longer than 6 months from the collection date prior to analysis. Patient age, body weight and current methylphenidate dose were recorded at the time of sample collection. Sample collection occurred about two months after treatment initiation for each patient, i.e. after dose titration. Patients were asked to consent to urine sampling without prior warning. Ethics approval for the study was granted to the NIHR Maudsley Biomedical Research Centre (Brain Connectivity in ADHD: a biomarker to predict treatment response; REC number 12/LO/0630).

On the day of sample collection, clinical response to stimulants was measured through the Global Clinical Impression Scale-Improvement (Busner and Targum, 2007; Guy, 1976) by a clinician blind to the assay results. The scale ranges from 1 (very much improved as compared to baseline) to 7 (very much worsened). The results were used to classify patients as 'responders' (≤ 2), 'mild responders' (3) and 'non-responders' (≥ 4). One-way ANOVA was used to measure group differences in creatinine-corrected concentration of (i) methylphenidate, (ii) ritalinic acid, and (iii) methylphenidate plus ritalinic acid. All samples were also screened for other stimulants (amfetamine, metamfetamine, MDMA, mephedrone, cocaine) using LC-HRMS (Chapter 2).

To enable summation of methylphenidate and ritalinic acid concentration, all patient results were converted to molar units (methylphenidate (µmol/L) = methylphenidate (µg/L)/233.32; ethylphenidate (µmol/L) = ethylphenidate (µg/L)/247.33; ritalinic acid (µmol/L) = ritalinic acid (µg/L)/219.28). Creatinine-corrected analyte concentration (µmol/mmol) was calculated by dividing the analyte concentration (µmol/L) by creatinine concentration (mmol/L).

4.3 Results and Discussion

4.3.1 LC-HRMS Method Validation

Intra- and inter-assay accuracy and precision are summarised in Table 4.1. The LoD was 0.002 mg/L and the LLoQ was 0.05 mg/L for all analytes. Typical chromatograms are shown in Figure 4.2. Typical mass accuracy was <1 ppm for all analytes (Table 4.2). No significant ion suppression or enhancement was observed, and the internal standards compensated well for matrix effects (Table 4.3). The mean linear regression coefficients (N = 6) obtained for methylphenidate, ethylphenidate, and ritalinic acid were 0.9999, 0.9993, and 0.9997, respectively. Recalculation of the calibrators demonstrated assay linearity with accuracy between 95-120 % (Table 4.4), and the assay was linear to at least 10 mg/L for all analytes (Figure 4.3). No significant carryover was observed at the concentrations studied (<1 % all analytes).

Table 4.1 – Methylphenidate, ethylphenidate, and ritalinic acid assay: Summary accuracy and precision data

	Analyte											
	Methylphenidate				**Ethylphenidate**				**Ritalinic acid**			
Intra-assay (N = 5)												
Nominal concentration (mg/L)	0.10	0.25	0.75	2.50	0.10	0.25	0.75	2.50	0.10	0.25	0.75	2.50
Measured concentration (mg/L)	0.10	0.25	0.74	2.41	0.09	0.25	0.78	2.41	0.11	0.24	0.77	2.55
% RSD	0.9	1.7	1.4	0.8	2.0	2.3	2.1	0.8	2.0	2.0	2.9	0.5
Accuracy (% nominal)	99	99	99	96	89	101	103	97	107	97	102	102
Inter-assay (singleton analysis, 5 separate days)												
Nominal concentration (mg/L)	0.10	0.25	0.75	2.50	0.10	0.25	0.75	2.50	0.10	0.25	0.75	2.50
Measured concentration (mg/L)	0.10	0.24	0.72	2.71	0.10	0.26	0.76	2.62	0.10	0.26	0.75	2.40
% RSD	14.0	10.8	7.7	7.8	12.3	6.3	7.9	5.3	19.6	5.5	7.4	4.2
Accuracy (% nominal)	98	96	96	109	96	103	101	105	97	104	100	96

Figure 4.2 – Methylphenidate, ethylphenidate, and ritalinic acid assay: Typical extracted ion chromatograms showing all analytes ($[M+H]^+$ ion *m/z* ±10 ppm) in a) the negative calibrator, b) the Limit of Detection (all analytes 0.002 mg/L), and c) Calibrator 2 (all analytes 0.5 mg/L)

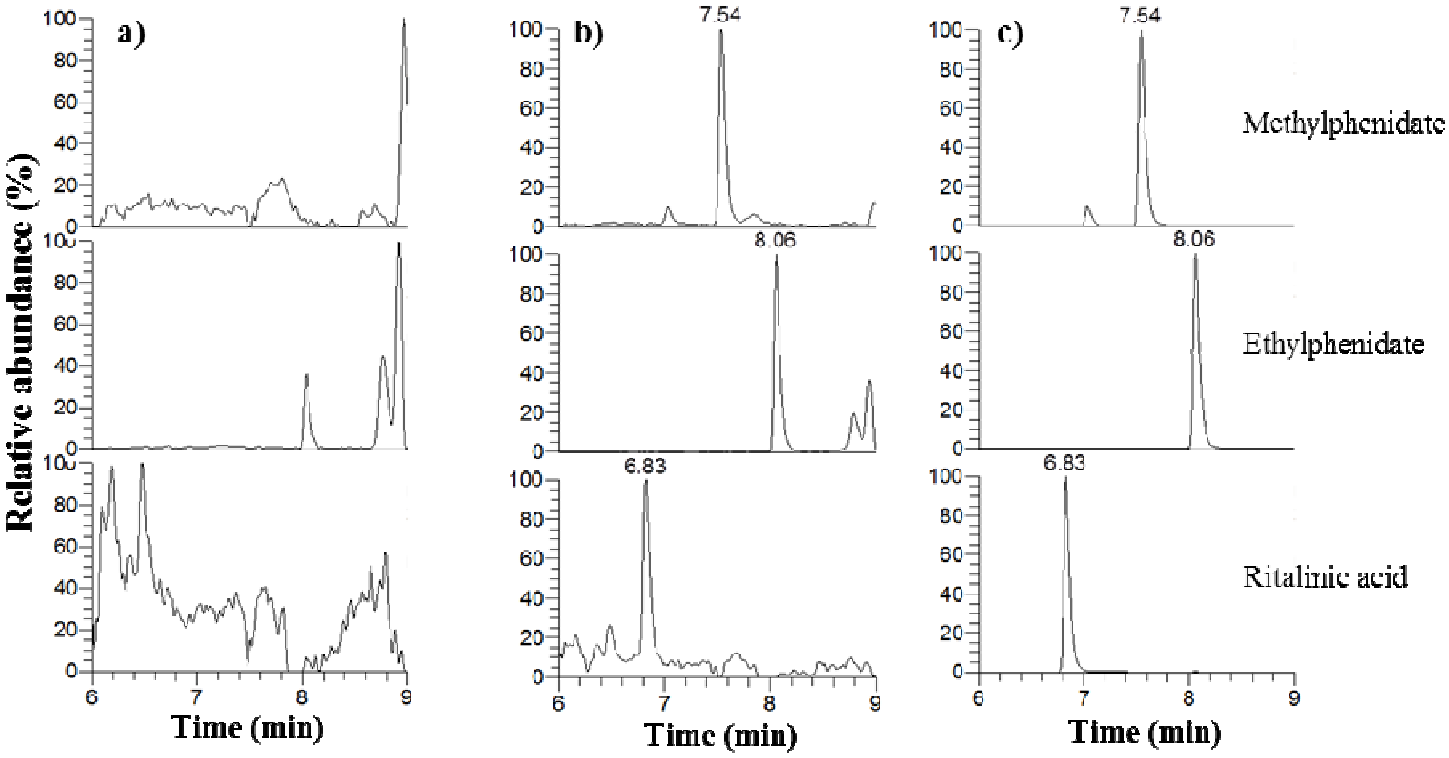


Table 4.2 – Methylphenidate, ethylphenidate, and ritalinic acid assay: Typical LC-HRMS data

Analyte	Mean (SD) retention time (min)	Theoretical $[M+H]^+$ (*m/z*)	Mean measured $[M+H]^+$ (*m/z*)	Mean delta *m/z* (ppm)	Product ion(s) (*m/z*)
Methylphenidate	7.55 (0.01)	234.1489	234.1490	0.43	84.0814 129.0700 174.1278
Ethylphenidate	8.07 (0.01)	248.1645	248.1646	0.40	84.0814 129.0706 174.1285
Ritalinic acid	6.88 (0.01)	220.1332	220.1333	0.45	84.0814 129.0700 174.1278
Methylphenidate-D_9	7.52 (0.01)	243.2054	243.2054	0.00	92.1316 93.1377 183.184
Ritalinic acid-D_{10}	6.85 (0.01)	230.1960	230.1959	-0.43	92.1316 93.1379 184.1904

Table 4.3 – Methylphenidate, ethylphenidate, and ritalinic acid assay: Summary matrix effects data

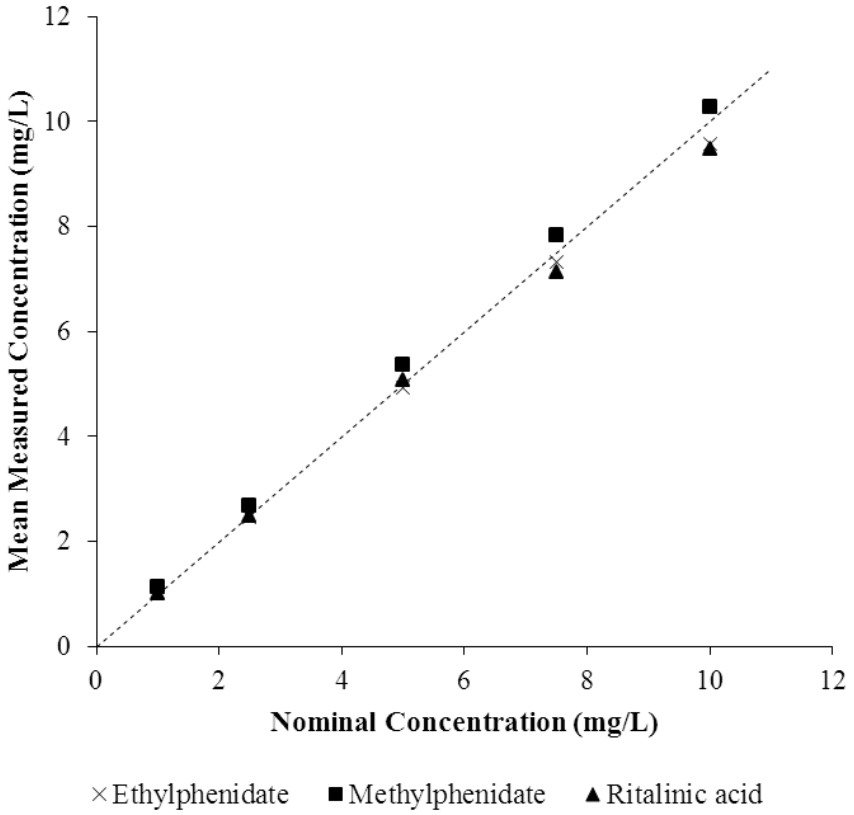
Solutions containing all analytes and internal standards were prepared in (i) deionised water, and (ii) analyte-free human urine from 10 independent sources. Prepared solutions were diluted (1+9, v/v) with eluent A and analysed in duplicate. The ratio of the peak area for each analyte to that of the relevant internal standard in analyte-free human urine samples was compared to that in deionised water.

Analyte	Mean Matrix Effect (%)	Relative matrix effect (%)
Methylphenidate-D_9	64	-
Methylphenidate	65	101
Ethylphenidate	65	101
Ritalinic acid-D_{10}	129	-
Ritalinic acid	128	99

Table 4.4 – Methylphenidate, ethylphenidate, and ritalinic acid assay: Summary linearity data

Analyte	Mean (range) accuracy (%)		
	Calibrator 1 0.05 mg/L	Calibrator 2 0.50 mg/L	Calibrator 3 5.00 mg/L
Methylphenidate	110 (109-112)	101 (100-102)	100 (99-102)
Ethylphenidate	119 (116-120)	103 (100-105)	99 (96-101)
Ritalinic acid	104 (102-105)	96 (95-99)	100 (98-102)

Figure 4.3 – Methylphenidate, ethylphenidate, and ritalinic acid assay: Linearity of the LC-HRMS Assay



4.3.2 Patient Results

The patient sample results are summarised in Table 4.5. Methylphenidate and ritalinic acid were detected in 18 and 24 samples, respectively. Ethylphenidate was detected in one sample (0.27 µmol/L) in addition to methylphenidate and ritalinic acid (6.20 and 95.7 µmol/L, respectively). No other stimulants were detected in any samples. Response to methylphenidate ranged from 1 to 4 (14 responders, 9 mild responders, 4 non-responders). Three samples had no methylphenidate, or ritalinic acid, detected. Two of these patients were classified as responders and one as a mild responder (Table 4.6).

Table 4.5 – Methylphenidate, ethylphenidate, and ritalinic acid assay: Summary patient data

MPH = methylphenidate, RA = ritalinic acid

	N	Mean	SD	Median	Minimum	Maximum
Age (y)	27	29	8	30	20	45
Daily dose (mg)	27	49	14	54	36	90
Body weight (kg)	27	81	12	80	54	102
Daily dose (mg/kg)	27	0.62	0.17	0.64	0.43	1
Urine creatinine (mmol/L)	27	15.4	9.3	13.2	1.4	40.5
Urine MPH (µmol/L)	18	2.50	2.55	1.69	0.27	10.5
Urine RA (µmol/L)	24	82.0	65.0	78.2	20.5	266
Urine MPH:RA ratio	18	0.029	0.018	0.033	0.003	0.069
Urine MPH + RA (µmol/L)	24	83.9	66.8	68.1	20.5	277

The creatinine-corrected urine methylphenidate, ritalinic acid and methylphenidate plus ritalinic acid concentrations compared to the clinical response achieved over the duration of methylphenidate therapy are summarised in Figure 4.4. There was no significant difference between the 3 response groups in the creatinine-corrected ritalinic acid concentration ($F(2,21) = 0.70$ $p = 0.51$) or creatinine-corrected methylphenidate plus ritalinic acid concentration ($F(2,21) = 0.81$ $p = 0.46$). However, the creatinine-corrected methylphenidate concentration was significantly different between the 3 groups ($F(2,16) = 37.53$, $p < 0.001$) with the median creatinine-corrected methylphenidate concentration being much higher in non-responders (clinical response 4) as compared to responders (clinical response 2/3). It should be noted that the trial participants had no prior warning of the request to provide a urine specimen, hence it might have been thought that 'topping up' with drug prior to the clinic visit to ensure detection in urine could be excluded, but this does seem nevertheless to be the most likely explanation for this result.

Table 4.6 – Methylphenidate, ethylphenidate, and ritalinic acid assay: Patient results

MPH = methylphenidate, ETH = ethylphenidate, RA = ritalinic acid

* Immediate release methylphenidate (Ritalin)

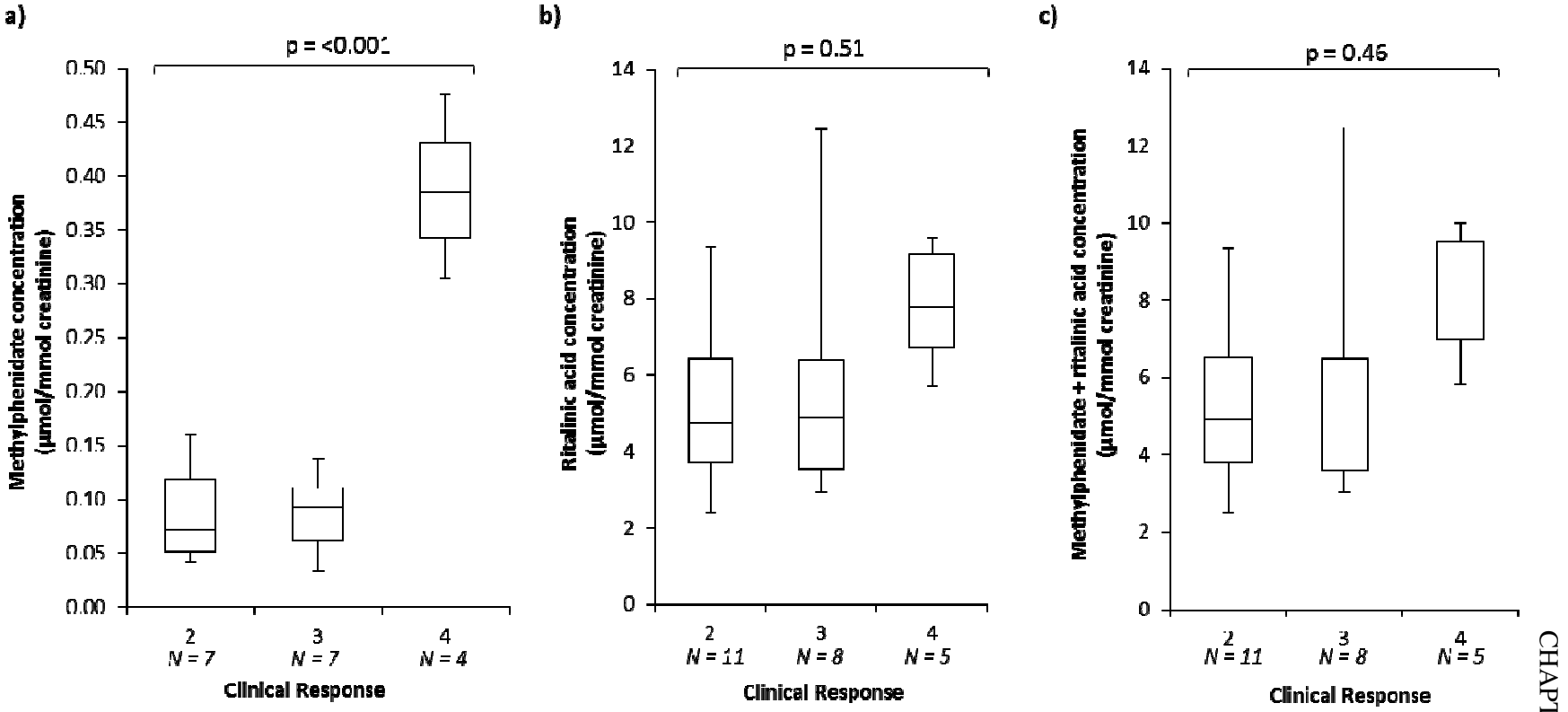
Sample ID	Age (y)	Dose (mg/d)	Clinical response score	Time since last dose (h)	Creatinine (mmol/L)	Analyte concentration (µmol/L)			Creatinine-corrected concentration (µmol/mmol)			MPH + RA (µmol/L)	Creatinine-corrected MPH + RA (µmol/mmol creatinine)
						MPH	ETH	RA	MPH	ETH	RA		
1	29	54	3	-	10.9	0.53	<0.20	89.1	0.05	-	8.17	89.6	8.22
2	38	54	4	-	12.3	6.20	0.27	95.7	0.50	0.02	7.78	101.9	8.29
3	45	54	2	2	11.6	0.46	<0.20	65.0	0.04	-	5.60	65.5	5.64
4	20	36	2	3	19.7	<0.21	<0.20	<0.23	-	-	-	-	-
5	29	18	3	5	13.2	<0.21	<0.20	<0.23	-	-	-	-	-
6	26	54	4	2	9.5	<0.21	<0.20	47.8	-	-	5.03	47.8	5.03
7*	22	20	3	3	1.4	<0.21	<0.20	31.3	-	-	22.36	31.3	22.4
8	18	54	2	3	3.8	<0.21	<0.20	35.6	-	-	9.36	35.6	9.36
9	32	54	2	3	10.8	<0.21	<0.20	<0.23	-	-	-	-	-
10	20	36	3	3	23	2.13	<0.20	77.6	0.09	-	3.37	79.8	3.47
11	22	36	2	3	12.2	1.00	<0.20	29.4	0.08	-	2.41	30.4	2.49
12	33	54	2	2	22.1	1.57	<0.20	148.8	0.07	-	6.73	150.4	6.81
13	34	54	2	2	14	<0.21	<0.20	70.8	-	-	5.06	70.8	5.06
14	20	36	2	2	21.1	1.26	<0.20	93.3	0.06	-	4.42	94.6	4.48
15	21	90	3	3	16.7	1.26	<0.20	32.0	0.08	-	1.91	33.2	1.99
16	30	54	3	2	25.2	0.27	<0.20	91.4	0.01	-	3.63	91.7	3.64

Table 4.6 (cont.) – Methylphenidate, ethylphenidate, and ritalinic acid assay: Patient results

MPH = methylphenidate, ETH = ethylphenidate, RA = ritalinic acid

Sample ID	Age (y)	Dose (mg/d)	Clinical response score	Time since last dose (h)	Creatinine (mmol/L)	Analyte concentration (µmol/L)			Creatinine-corrected concentration (µmol/mmol)			MPH + RA (µmol/L)	Creatinine-corrected MPH + RA (µmol/mmol creatinine)
17	40	36	2	-	9.4	0.40	<0.20	32.6	0.04	-	3.47	33.0	3.51
18	24	54	4	3	6.1	1.71	<0.21	41.0	0.28	-	6.71	42.7	6.99
19	21	54	3	2	10	1.67	<0.21	40.7	0.17	-	4.07	42.4	4.24
20	34	36	2	3	16.9	2.80	<0.21	80.3	0.17	-	4.75	83.1	4.92
21	34	54	2	3	19.9	3.09	<0.21	78.7	0.16	-	3.96	81.8	4.11
22	22	54	3	2	40.5	4.80	<0.21	229.7	0.12	-	5.67	234.5	5.79
23	35	72	3	3	32.6	3.37	<0.21	189.7	0.10	-	5.82	193.0	5.92
24	29	54	4	-	29.1	10.5	<0.21	266.1	0.36	-	9.15	276.7	9.51
25	42	54	2	2	3.6	<0.21	<0.21	36.5	-	-	10.14	36.5	10.1
26	29	54	4	3	4.5	1.83	<0.21	44.5	0.41	-	9.89	46.3	10.3
27	23	54	2	2	15.1	<0.21	<0.21	20.5	-	-	1.35	20.5	1.35

Figure 4.4 – Box and whisker plots (median, 25th-75th percentiles, whiskers 10th and 90th percentiles) to show the measured urinary concentration of a) methylphenidate (µmol/mmol creatinine), b) ritalinic acid (µmol/mmol creatinine), and c) methylphenidate + ritalinic acid (µmol/mmol creatinine) in urines from patients in each clinical response category



The presence of ethylphenidate in the urine of one patient may be a result of direct ethylphenidate ingestion, or as a result of co-ingestion of methylphenidate with ethanol. Ethylphenidate formation has been reported *in vitro* using a rat liver preparation incubated with methylphenidate and ethanol (Bourland *et al.*, 1997). This biotransformation appears to be a carboxylesterase-dependent transesterification process, and may be analogous to that involved in the formation of cocaethylene (benzoylecgonine ethyl ester) by human hepatic esterase(s) after concomitant cocaine and ethanol use (Boyer and Petersen, 1992). The presence of ethylphenidate in addition to methylphenidate in urine can function as a marker for clinical or forensic evidence of concomitant methylphenidate-ethanol exposure (Patrick *et al.*, 2014).

4.3.3 Differentiating Ethylphenidate and Methylphenidate Administration

As regards distinguishing ethylphenidate from methylphenidate ingestion, methylphenidate is not a metabolite of ethylphenidate and thus should not be present in urine after use of ethylphenidate. Analysis of the urine sample from an individual suspected of taking ethylphenidate contained 10.3 µmol/L ethylphenidate and 108 µmol/L ritalinic acid (methylphenidate not detected). No other drugs of abuse were detected. The high ethylphenidate concentration combined with the absence of methylphenidate is strongly suggestive that ethylphenidate, and not methylphenidate in conjunction with ethanol, had been ingested. The mean proportions of methylphenidate, ethylphenidate, and ritalinic acid excreted in urine (0-6 h post dose) after administration of 20 mg methylphenidate with ethanol (0.6 g/kg consumed 30 min after methylphenidate) were calculated as 1.4 ±0.8, 0.02 ±0.1, and 19.9 ±10.8 % of the methylphenidate dose respectively (Markowitz *et al.*, 2000). This study concluded that the low concentration of ethylphenidate detected indicates that a single clinically relevant dose of methylphenidate in combination with moderate intake of ethanol is unlikely to result in substantial generation of ethylphenidate, as with the patient in this study. However, if only ritalinic acid is detected in urine distinguishing between use of methylphenidate, ethylphenidate, or other phenidate analogues is not possible on the basis of the analytical result alone.

4.4 Conclusions

One of the key advantages of HRMS is the flexibility to add drugs and/or their metabolites quickly to an existing method. Through keeping sample preparation as non-selective as possible (i.e. dilution) followed by collection of full scan MS data, addition of analytes is facilitated by simply adding their accurate molecular masses into processing software, provided the analyte is retained on the LC column and calibrators can be prepared. The incorporation of new analytes into the method is therefore without detriment to the original panel of analytes as no changes have been made to the analysis, only to data processing.

Urinary methylphenidate, ethylphenidate, and/or ritalinic acid analysis can help identify non-adherence to methylphenidate in the clinical setting, and also detect illicit use of methylphenidate and ethylphenidate. Ingestion of ethylphenidate results in a high urinary ethylphenidate concentration and the absence of methylphenidate, and can be differentiated from ethylphenidate arising from methylphenidate ingestion in conjunction with ethanol.

4.4.1 Further Work

The method could be expanded to include other phenidate analogues that are marketed as NPS (e.g. isopropylphenidate). However, the use and availability of ethylphenidate appear to have declined over recent years and this may mean that other phenidate analogues are also no longer in circulation.

5 Retrospective analysis of LC-HRMS data: Mephedrone

5.1 Introduction

5.1.1 Mephedrone

Mephedrone (4-methylmethcathinone, 4-MMC) is a β-ketoamfetamine that has stimulant properties similar to those of other amfetamines (e.g. amfetamine, metamfetamine, MDMA), as well as causing hallucinations and other psychotropic effects (Busardò *et al.*, 2015). Mephedrone contains a chiral centre and thus exists as *S*- and *R*-mephedrone. Both enantiomers have similar potency at dopamine transporters, but different potency at serotonin transporters with *R*-mephedrone lacking serotonergic actions (Gregg *et al.*, 2015). The clinical features of mephedrone intoxication are consistent with an acute sympathomimetic toxidrome, i.e. tachycardia, hypertension, and agitation (Adamowicz *et al.*, 2013; Cosbey *et al.*, 2013; Dickson *et al.*, 2010; Gerace *et al.*, 2014; Lusthof *et al.*, 2011; Wood *et al.*, 2010a, 2010b). Mephedrone is available in either tablet or powder form, with the predominant routes of use through either nasal insufflation, or ingestion (Dargan *et al.*, 2010; Winstock *et al.*, 2011). Injection of mephedrone has also been reported (Hope *et al.*, 2016; Péterfi *et al.*, 2014).

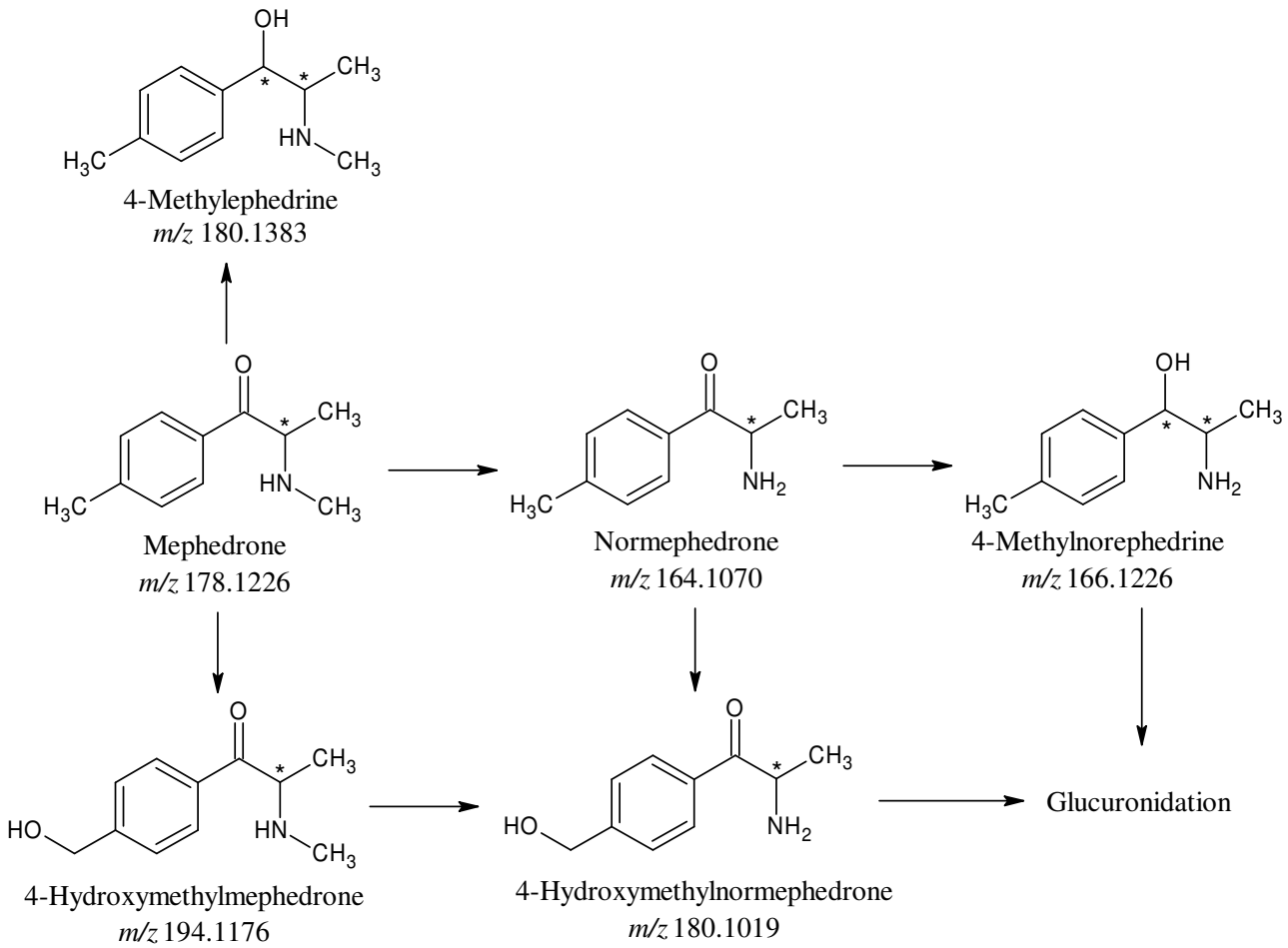
In 2010, mephedrone was scheduled as a Class B substance under the MDA within the UK, and by the end of 2010 mephedrone had been controlled across much of Europe (Kelly, 2011). Since 2010, mephedrone use has persisted, in particular amongst 16-24 year olds, though such use has been declining (falling from 4.4 to 0.9 % among 16-24 year olds from 2010 to 2016; Home Office, 2013-2016). The use of mephedrone is reported to be more common in males than females (Vardakou *et al.*, 2011).

5.1.2 Mephedrone Metabolism

There is limited information regarding the pharmacokinetics of mephedrone, although metabolism has been extensively studied in rat hepatocytes (Khreit *et al.*, 2013), and in human liver microsomes (Pedersen *et al.*, 2013), as well as *in vivo* in rats (Meyer *et al.*, 2010) and humans (Pozo *et al.*, 2015). CYP2D6 has been identified as the main enzyme responsible for the phase I *in vitro* metabolism of mephedrone (Pedersen *et al.*, 2013). Mephedrone may be metabolised to *N*-desmethylmephedrone (normephedrone), β-hydroxynormephedrone (4-methylnorephedrine), 4-hydroxymethylmephedrone, and 4-hydroxymethylnormephedrone, with further conjugation of the hydroxylated metabolites (Meyer *et al.*, 2010, Figure 5.1). Large inter-subject variability in metabolism has been reported, which may be due to either genetic variation in CYP2D6 activity, or as a result of low oral bioavailability (Olesti *et al.*, 2017).

Figure 5.1 – Proposed metabolic pathway of mephedrone in humans, Meyer *et al.* (2010)

Theoretical exact mass given for each compound; * chiral centre



5.1.3 Reported Mephedrone Concentrations in Biological Matrices

The mean (range) post-mortem whole blood mephedrone concentration in 10 adults who died of acute mephedrone overdose was 4.5 (0.5-22) mg/L (Adamowicz *et al.*, 2013; Aromatario *et al.*, 2012; Cosbey *et al.*, 2013; Maskell *et al.*, 2011a; Rojek *et al.*, 2014; Torrance and Cooper, 2010). The mean (range) blood mephedrone concentration detected in 32 impaired driving cases (mephedrone was the only drug detected in 9 cases) was 0.21 (0.01-0.74) mg/L (Cosbey *et al.*, 2013).

Concheiro *et al.* (2015) screened urine samples from stimulant users for NPS and reported mephedrone, normephedrone, and 4-methylephedrine concentrations ranging from 0.012-3.60 mg/L (N = 11), 0.004-8.79 mg/L (N = 12), and 0.011-0.33 mg/L (N = 6), respectively. Single case reports measured urinary mephedrone concentrations of 16 mg/L in a known drug user (Hong *et al.*, 2016), and 198 mg/L post-mortem from a case of accidental death attributed to multiple-drug toxicity of mephedrone and heroin (Dickson *et al.*, 2010). Mephedrone has been shown to be stable in plasma and urine stored at -20 ^{o}C for at least 6 months (Olesti *et al.*, 2017).

5.1.4 Retrospective Data Interrogation

The ability to reprocess data acquired during routine drugs of abuse screening to search for compounds that were not targeted initially can help improve knowledge of the metabolism of novel compounds. Analytes may also be quantified retrospectively through subsequent analysis of calibrator solutions containing the same internal standard (at the same concentration) added at the time of original analysis. The internal standard should largely account for any difference in instrument response between the initial analysis data and the later analysis of the calibration solutions.

5.1.5 Aims

This chapter aims to investigate another advantage of LC-HRMS, the ability to perform retrospective data interrogation. Recently, reference standards for some metabolites of mephedrone have become available commercially. Given the limited data on mephedrone and mephedrone metabolite urinary concentrations, data resulting from routine drug screening analyses where mephedrone was detected will be retrospectively analysed. In addition, the cross-reactivity of mephedrone and selected metabolites will be ascertained using a commercially available amfetamine-group immunoassay to assess the suitability of the assay for detecting the use of mephedrone.

5.2 Materials and Methods

5.2.1 Chemicals and Reagents

Mephedrone hydrochloride, buphedrone hydrochloride, and methcathinone hydrochloride were supplied as methanolic solutions (all 1 mg/mL free base) from Cerilliant (Sigma, Poole, UK). Normephedrone hydrochloride, 4-methylephedrine hydrochloride, and 4-methylpseudoephedrine hydrochloride were supplied as powders from Cayman Chemicals (Michigan, USA). Hydrochloric acid was from Sigma-Aldrich (Poole, UK). For all other chemicals and reagents see Section 2.2.1.

5.2.2 Instrumentation

All instrumentation is detailed in Section 2.2.2.

5.2.3 Preparation of Calibration and Internal Quality Control Solutions

Normephedrone, 4-methylephedrine, and 4-methylpseudoephedrine calibration and IQC stock solutions (1,000 mg/L free base) were prepared separately in aqueous hydrochloric acid (0.1 mol/L). Mephedrone (1,000 mg/L in methanol) was used as supplied. Separate calibration and IQC working solutions (5 mg/L, all analytes) were prepared by appropriate dilution of stock and reference solutions in volumetric glassware with aqueous hydrochloric acid (0.1 mol/L).

Calibrators (0.01-1.0 mg/L, all analytes) and IQC solutions (0.02 and 0.25 mg/L, all analytes) were prepared by dilution of the stock or working solutions with analyte-free human urine. After thorough mixing and standing overnight (2-8 ºC), 1 mL portions of all solutions were transferred to 2 mL screw-cap polypropylene tubes, which were then capped and stored at -20 ºC until required.

5.2.4 Internal Standard Solution

IS solution was prepared as described in Section 2.2.7, and used for analysis of the mephedrone calibrators, IQC solutions, and re-analysis of patient samples. Mephedrone-D_3 was not included in the IS solution at the time of the original sample analysis, so codeine-D_6 was selected for the retrospective analysis.

5.2.5 Sample Preparation

Samples were prepared as described in Section 2.2.9.

5.2.6 LC-HRMS Method

The LC-HRMS method (Chapter 2) was used for the original analysis of patient samples, and for subsequent analysis of samples, calibrator and IQC solutions.

To ascertain the suitability of the method for the detection of urinary mephedrone metabolites, a brief validation study was conducted. Intra- and inter-assay accuracy and precision were measured by replicate analysis (N = 5) of the IQC solutions on the same day and by singlicate analysis on different days (N = 3), respectively. The LoD was ascertained through analysis of the low calibrator and was based upon a signal-to-noise ratio >3, and the RSD <20 % (N = 5). To investigate matrix effects, solutions containing all analytes at 0.2 mg/L were prepared in (i) analyte-free human urine from 10 independent sources, and (ii) eluent A. Prepared solutions were diluted (1+9, v/v) with eluent A and analysed. The ratio of the peak area of each analyte to that of codeine-D_6 and mephedrone-D_3 was compared for each analyte in the presence and absence of matrix. To investigate isobaric interferences, aqueous solutions of buphedrone and methcathinone (both 1 mg/L free base) were analysed to ascertain retention times and confirm the MS^2 product ions.

5.2.7 Assay Calibration and Acceptance Criteria

5.2.7.1 LC-HRMS

Calibration standards (N = 4), IQC solutions (N = 2), and a matrix blank (analyte-free human urine) were analysed up to 1.1 years after the original analyses. Assay acceptance criteria were (i) linear (R^2 >0.98) calibration curves for each analyte, and (ii) IQC samples within 20 % of their respective nominal value.

Peak area ratios (analyte to IS) obtained on analysis of the calibration standards were plotted against concentration to construct calibration graphs. Linear regression intercepts were not forced through zero, and no weighting was applied.

5.2.7.2 CEDIA

The assays were calibrated as necessary using the calibrators (*S*-metamfetamine 0, 0.5, 1, 3 and 5 mg/L for amfetamine group assay; creatinine 0.18 and 1.8 mmol/L) supplied with the assay kits. IQC solutions were analysed prior to the analysis of patient samples.

5.2.8 Assessment of CEDIA Cross-reactivity

Mephedrone, normephedrone, 4-methylephedrine, and 4-methylpseudoephedrine were added to separate portions of analyte-free human urine at concentrations of 10, 20, and

50 mg/L (free base), and analysed in duplicate using the amfetamine-group CEDIA. The mean measured concentration of the 50 mg/L solution was used to calculate the relative cross-reactivity of each analyte with the assay [(mean measured concentration/nominal concentration) x 100 %].

5.2.9 Patient Samples

Data from urine samples analysed for routine clinical drug screening between March 2015-January 2016 inclusive that had a detectable mephedrone concentration (>0.01 mg/L) were analysed retrospectively for the presence of selected metabolites. Normephedrone, 4-methylephedrine, and 4-pseudomethylephedrine concentrations were estimated by comparison with the results of the analysis of calibrators prepared and assayed a maximum of 1.1 years after the original sample analysis. To assess the accuracy of the concentrations ascertained by retrospective analysis, all patient urine samples (stored at -20 ^{o}C since original analysis) were re-analysed with the prepared calibrators. On re-analysis, samples were analysed undiluted and after appropriate dilution (using analyte-free urine) to bring analyte concentrations within the calibration range. For samples where mephedrone was detected by LC-HRMS, analysis using the amfetamine-group CEDIA was also performed when the sample was originally received in the laboratory.

Data analysis was performed by using Microsoft Excel 2010 (version 14.0.7166.5000) and Analyse-It (version 2.3). The Shapiro-Wilk test was used to test normality of data sets. For non-parametric data, the Mann-Whitney U test was used to compare data sets.

To enable comparison of the proportions of mephedrone and each metabolite present in urine samples, all concentrations were converted to µmol/L (Equation 5.1).

<u>Equation 5.1</u> – Conversion of Analyte Concentration from µg/L to µmol/L

Analyte molecular weight: mephedrone – 177.1154, normephedrone – 163.0997, 4-methylephedrine/4-methylpseudoephedrine – 179.1310

$$Analyte\ concentration\ (\mu mol/L) = \frac{Analyte\ concentration\ (\mu g/L)}{Analyte\ molecular\ weight}$$

5.2.10 Ethics

All patient samples were submitted to the laboratory for urine drug screening. Excess sample was used for this work, with all samples anonymised prior to additional investigation.

5.3 Results and Discussion

5.3.1 CEDIA Cross-reactivity

The analysis of analyte-free human urine to which mephedrone, normephedrone, 4-methylephedrine, or 4-methylpseudoephedrine had been added (each 50 mg/L free base) showed a cross-reactivity of 0.9, 0.2, 0.6, and 0.6 %, respectively with the amfetamine-group CEDIA. It was thus calculated that separate urinary mephedrone, normephedrone, 4-methylephedrine, and 4-methylpseudoephedrine concentrations of 57, 250, 78, and 81 mg/L, respectively, would be required to produce a positive amfetamine-group CEDIA result of 0.5 mg/L (Figure 5.2).

5.3.2 Ascertaining LC-HRMS Assay Suitability

Intra- and inter-assay accuracy and precision are summarised in Table 5.1. Typical chromatograms are shown in Figure 5.3. The LoD was 0.01 mg/L for all analytes (RSD was <4 % for all analytes).

Of the internal standards present at the time of original analysis, codeine-D_6 was selected for retrospective analysis as it had the closest retention time to the analytes of interest. Matrix effects were investigated for both mephedrone-D_3 and codeine-D_6 to ascertain their suitability as internal standards. Mephedrone-D_3 compensated well for matrix effects for all analytes, with RSD <20 % for all analytes among the 10 different urines. Codeine-D_6 did not compensate as well showing significant variation between the 10 matrices (RSD >20 % for all analytes), however for retrospective analysis was deemed adequate (Table 5.2).

Figure 5.2 – Cross-reactivity of mephedrone, normephedrone, 4-methylephedrine, and 4-methylpseudoephedrine with the amfetamine-group CEDIA (see Section 5.3.1)

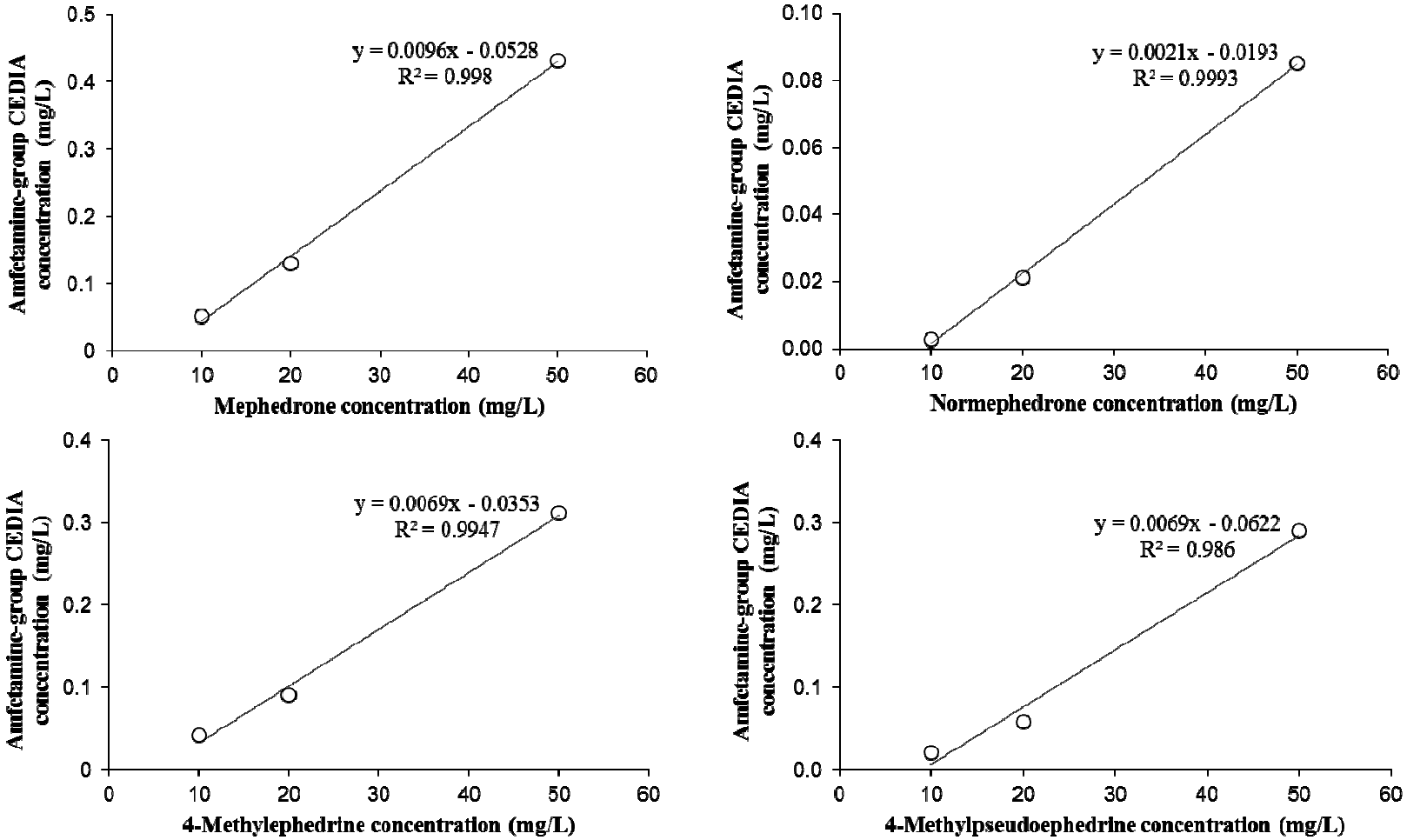


Table 5.1 – Mephedrone assay: Intra- and Inter-assay accuracy and precision data

Accuracy (% nominal IQC value) and precision (% RSD)

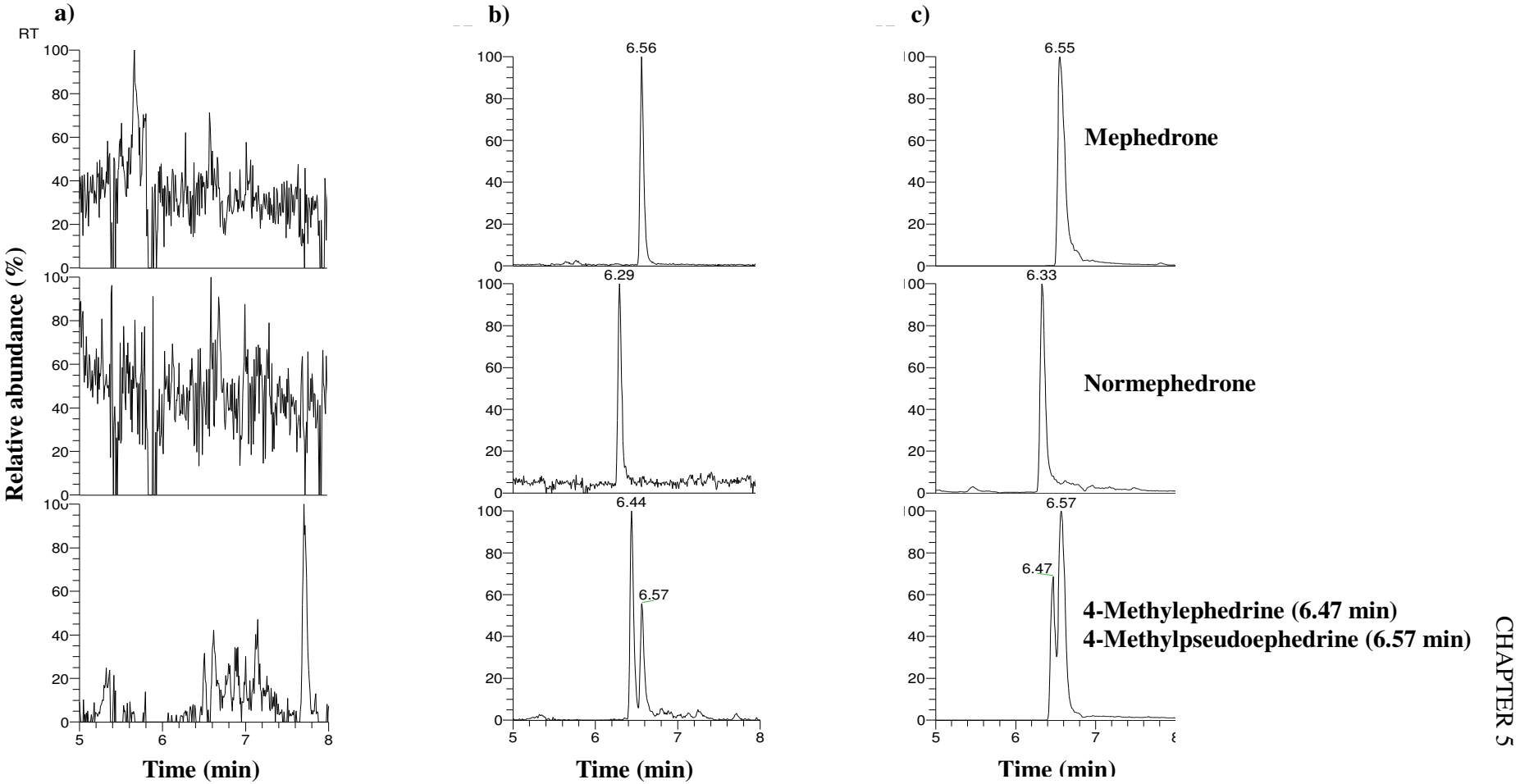
Analyte	Nominal (mg/L)	Mean measured (mg/L)	RSD (%)	Accuracy (%)
Intra-assay (N = 5)				
Mephedrone	0.02 0.25	0.02 0.24	0.7 0.8	93 97
Normephedrone	0.02 0.25	0.02 0.24	7.4 1.6	81 95
4-Methylephedrine	0.02 0.25	0.02 0.25	4.8 2.7	96 98
4-Methylpseudoephedrine	0.02 0.25	0.03 0.23	1.7 1.0	106 90
Inter-assay (N = 3, separate days)				
Mephedrone	0.02 0.25	0.02 0.26	3.3 4.0	94 106
Normephedrone	0.02 0.25	0.02 0.23	7.1 5.8	80 92
4-Methylephedrine	0.02 0.25	0.02 0.26	6.3 7.0	97 104
4-Methylpseudoephedrine	0.02 0.25	0.03 0.27	5.4 2.2	104 107

Table 5.2 – Mephedrone assay: Summary matrix effects data

Solutions containing all analytes and internal standards (0.2 mg/L free base) were prepared in (i) analyte-free human urine from 10 independent sources, and (ii) eluent A. Prepared solutions were diluted (1+9, v/v) with eluent A and analysed. The peak area of each analyte/internal standard was compared in the presence and absence of matrix to calculate the matrix effect. The ratio of the peak area of each analyte to that of the relevant internal standard was compared for each analyte in the presence and absence of matrix to calculate the relative matrix effect.

Analyte	Mean Matrix Effect (%)	Mephedrone-D_3		Codeine-D_6	
		Relative Matrix Effect (%)	RSD (%)	Relative Matrix Effect (%)	RSD (%)
Mephedrone	41	101	2	125	28
Normephedrone	52	130	8	161	25
4-Methylephedrine	35	87	16	112	29
4-Methylpseudoephedrine	43	109	18	128	38
Mephedrone-D_3	40	-	-	-	-
Codeine-D_6	38	-	-	-	-

Figure 5.3 – Extracted ion chromatograms of mephedrone, normephedrone, 4-methylephedrine, and 4-methylpseudoephedrine in a) analyte-free urine, b) the low calibrator (all 0.01 mg/L), and c) a patient sample (measured concentrations: mephedrone 6.80 mg/L; normephedrone 0.77 mg/L; 4-methylephedrine 1.41 mg/L; 4-methylpseudoephedrine 4.76 mg/L)



5.3.2.1 Isobaric Compounds

Where possible, compounds known to be isobaric with mephedrone and its metabolites were analysed to ensure the compound of interest could be distinguished from potential sources of interference. LC resolution was achieved for the isobaric compounds tested, but different MS^2 product ions could not be identified for all compounds (Table 5.3). Sufficient chromatographic resolution of 4-methylephedrine and 4-pseudo-methylephedrine was observed at the concentration tested (1 mg/L both analytes). However, at higher concentration chromatographic resolution was lost (Figure 5.4). Samples with 4-methylephedrine and 4-methylpseudoephedrine concentration greater than 1 mg/L should thus be diluted with analyte-free urine to enable accurate detection and quantification of these analytes.

Interference with the internal standard (mephedrone-D_3, *m/z* 181.1415) was observed as a result of the naturally-occurring isotope (M+1) of 4-methylephedrine and of 4-methylpseudoephedrine (both *m/z* 181.1416). Whilst these compounds are not isobaric, mass resolution was not possible as the compounds only differ by 0.0001 amu. 4-Methylpseudoephedrine co-elutes with mephedrone-D_3 which resulted in falsely elevated mephedrone-D_3 peak area as 4-methylpseudoephedrine concentration increased (Figure 5.5). Mephedrone-D_3 is therefore not an ideal internal standard for this assay. However, no other stable isotope-labelled analogues of mephedrone were commercially available at the time of re-analysis.

Table 5.3 – Chromatographic resolution and MS product ions for some compounds isobaric with mephedrone/mephedrone metabolites

[#] Ions resulting from a water loss excluded
[*] No reference compound available to analyse

Analyte	Molecular formula	Precursor ion (*m/z*)	Product ions[#] (*m/z*)	Retention time (min)
Mephedrone	$C_{11}H_{15}NO$	178.1226	145.0886 119.0855	6.27
2-Methylmethcathinone (2-MMC)			145.0886 119.0855	*
3-Methylmethcathinone (3-MMC)			145.0886 119.0855	*
Ethcathinone			132.0808 131.0730	*
Buphedrone			131.0730 132.0808	5.85
5-(2-Aminopropyl)-2,3-dihydrobenzofuran (5-APDB)			133.0648 161.0961	*
Normephedrone	$C_{10}H_{13}NO$	164.1070	147.0804 119.0855	6.30
Methcathinone			131.0730 105.0699	5.36
4-Methylephedrine	$C_{11}H_{17}NO$	180.1383	147.1028 131.0855	6.44
4-Methylpseudoephedrine			147.1028 131.0855	6.57
N-Methylephedrine/ *N*-Methylpseudoephedrine			147.1043 131.0855	*
Buphedrine			133.0886 131.0855	*

Figure 5.4 – Extracted ion chromatogram (*m/z* 180.1383) to show the influence of increasing the concentration of 4-methylephedrine and 4-pseudomethylephedrine (1-100 mg/L both analytes) on the chromatographic resolution of these compounds

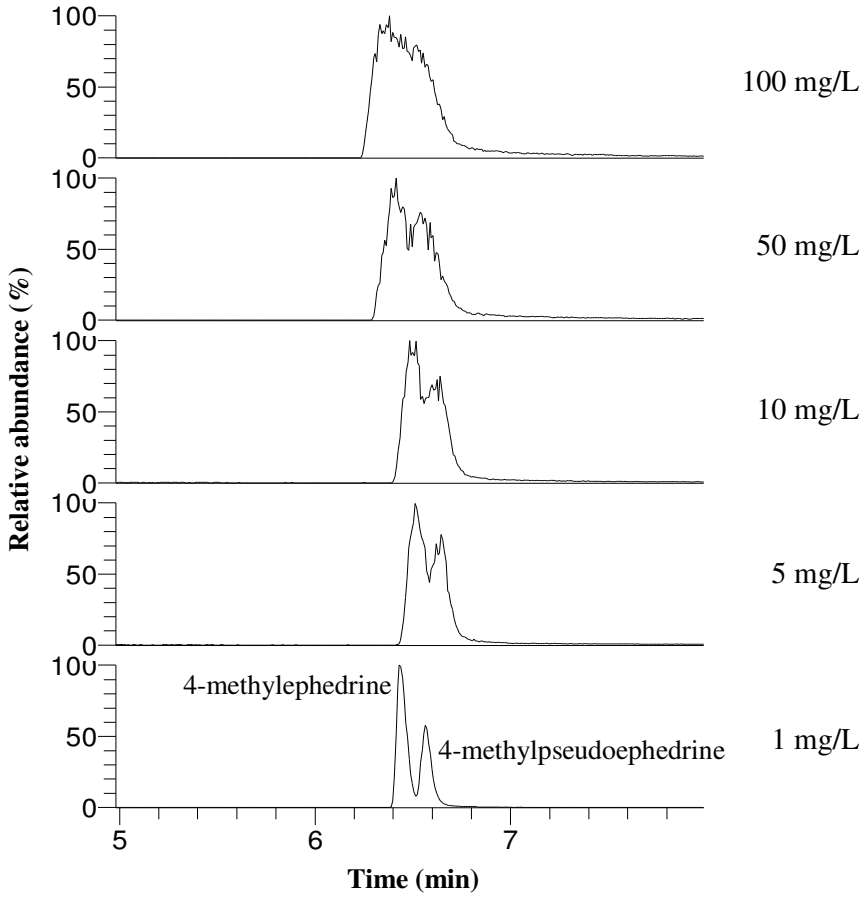
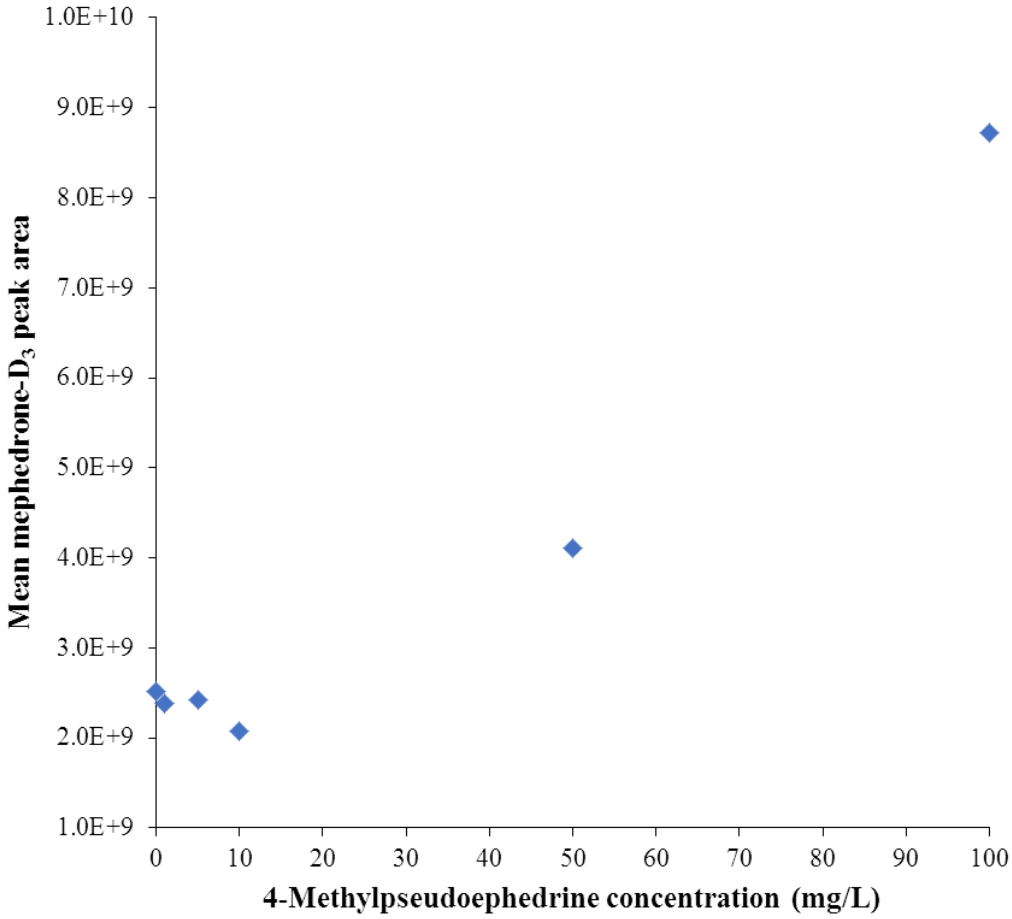


Figure 5.5 – The effect of 4-methylpseudoephedrine concentration on the measured mephedrone-D_3 peak area

Mean peak area (N = 2) for mephedrone-D_3 was compared in urine samples containing 4-methylpseudoephedrine (0.01-100 mg/L).



5.3.3 Assessing the Accuracy of Data Generated from Retrospective Analysis

Comparison of the calculated concentrations from re-analysis of undiluted samples using mephedrone-D_3 as an internal standard to those calculated from retrospective data analysis using codeine-D_6 as the internal standard showed poor agreement for all of the metabolites (Table 5.4, full data in Appendix A). Whilst the overall agreement between mephedrone concentrations calculated using mephedrone-D_3 and codeine-D_6 was good (R^2 = 0.91), discrepancies were apparent when mephedrone concentration was >10 mg/L. This may be due to the higher concentrations of 4-methylephedrine and 4-methylpseudoephedrine in these latter samples giving rise to interference with mephedrone-D_3 (see Section 5.3.1.1). For all other analytes, the calculated concentrations using codeine-D_6 as the internal standard were generally lower than when mephedrone-D_3 was used. This negative bias was more apparent at higher analyte concentration (>2 mg/L all analytes), which may be due to loss of chromatographic resolution between 4-methylephedrine and 4-methylpseudoephedrine causing inaccurate peak integration, and the resulting interference with mephedrone-D_3. Most samples (76 %) were stored frozen for longer than 6 months. As no stability data exists past this time frame for mephedrone, and none is published for the other analytes, it is possible that the analytes may have degraded since the time of the original analysis, contributing to the lower concentrations found on re-analysis.

The re-analysis of samples after appropriate dilution showed that the measured concentrations from retrospective analysis (where samples were analysed undiluted) were likely underestimating the concentration of all the analytes studied (Table 5.5, full data in Appendix B). This underestimation is likely due to the interference from the ^{13}C-isotopes of 4-methylephedrine and 4-methylpseudoephedrine that caused the measured peak area of mephedrone-D_3 to be falsely elevated. The increased internal standard peak area caused the peak area ratio of the analyte to internal standard to be reduced, resulting in a lower calculated analyte concentration.

Table 5.4 – Characteristics of x-y and Bland-Altman plots comparing the measured analyte concentration when using codeine-D_3, as opposed to mephedrone-D_3, as the internal standard

NB. Full data is given in Appendix A

Analyte	R^2	y-intercept	Slope	Bias
Mephedrone	0.9115	2.20	0.95	1.67
Normephedrone	0.7477	0.20	0.42	-2.50
4-Methylephedrine	0.8271	0.27	0.38	-0.82
4-Methylpseudoephedrine	0.5956	0.75	0.23	-1.94

Table 5.5 – Characteristics of x-y and Bland-Altman plots comparing the measured analyte concentration from diluted urine samples as opposed to undiluted urine samples

NB. Full data is given in Appendix B

Analyte	R^2	y-intercept	Slope	Bias
Mephedrone	0.7931	-13.4	4.2	27.3
Normephedrone	0.5422	0.18	1.6	1.44
4-Methylephedrine	0.5184	-0.47	2.4	0.95
4-Methylpseudoephedrine	0.5735	-1.52	3.6	2.53

5.3.4 Interpretation of Results from Analysis of Patient Samples

Twenty-five mephedrone-containing urine samples from 24 patients were analysed retrospectively for the presence of mephedrone metabolites. Nineteen samples were from males [median (range) age: 37 (22-58) y], and six from females [median (range) age: 32 (21-49) y]. Samples were received from community drug and alcohol rehabilitation teams (N = 10), referrals from other hospital laboratories (N = 11), general practitioners (N = 2), and critical care wards (N = 2). The reason for request was given for 16 samples, of which 10 were for routine monitoring as part of drug rehabilitation treatment and 6 were querying drug overdose (mephedrone specified in 2 cases).

5.3.4.1 Quantitative Data from LC-HRMS Analysis

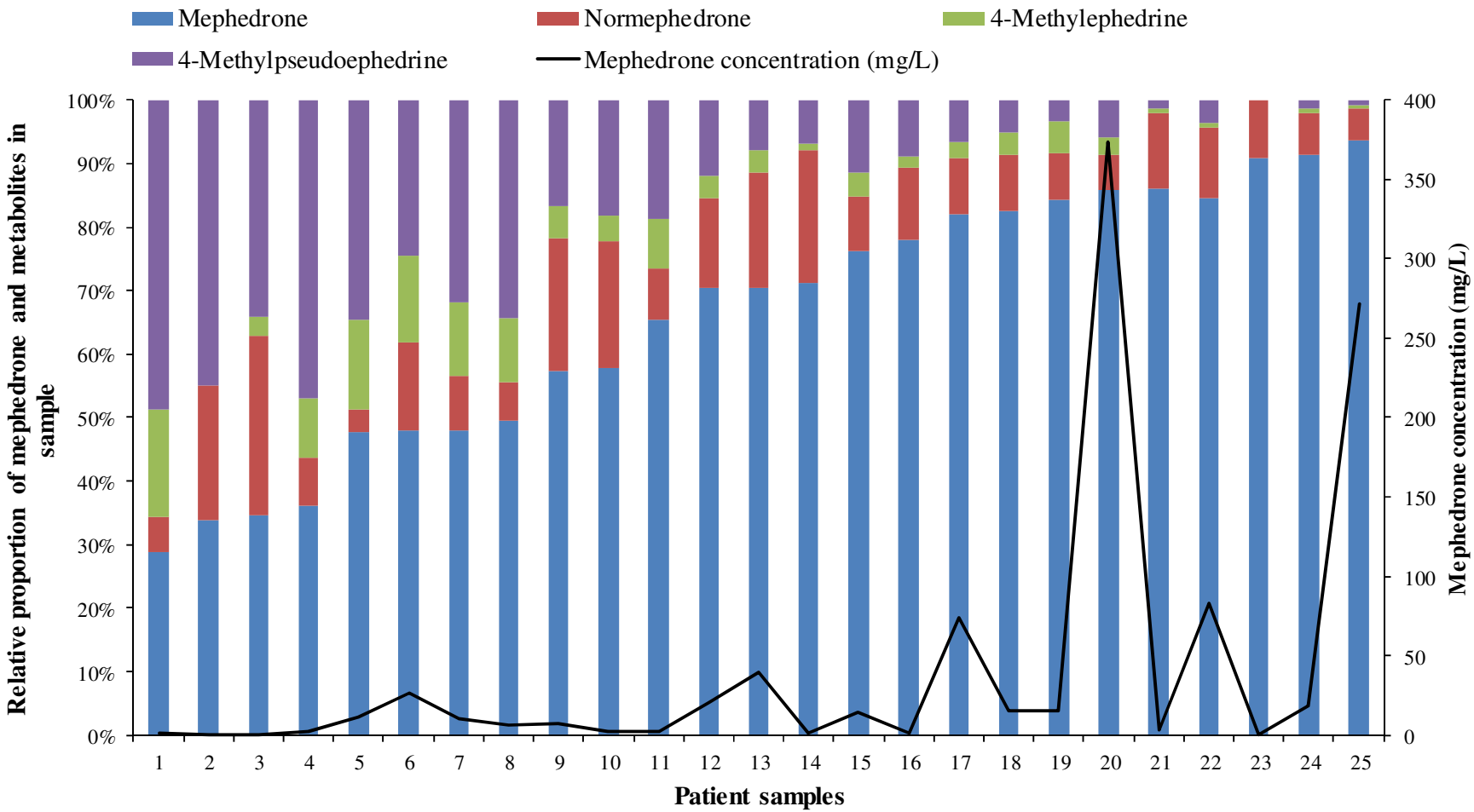
Due to the problems associated with retrospective analysis of samples mentioned in Section 5.3.2, the concentrations ascertained on re-analysis of urine samples using mephedrone-D_3 as an internal standard (and after appropriate dilution where necessary) were deemed more accurate and are summarised in Table 5.6. Most samples (60 %) had other amfetamine-type drugs (amfetamine, metamfetamine, or MDMA) detected in addition to mephedrone.

In the majority of the urine samples, mephedrone was the predominant species detected (Figure 5.6). No information was available regarding the time of mephedrone ingestion in relation to the time of sampling.

Table 5.6 – Summary of mephedrone and metabolite concentration in urine samples on re-analysis using mephedrone-D_3 as an internal standard, and after appropriate sample dilution where necessary

Analyte	N	Concentration (mg/L)			
		Mean	Minimum	Median	Maximum
Mephedrone	25	40.1	0.05	10.5	373.6
Normephedrone	25	3.5	0.01	1.2	22.2
4-Methylephedrine	23	1.7	0.02	0.7	11.9
4-Methylpseudoephedrine	24	3.9	0.06	2.2	26.3

Figure 5.6 – Relative proportion of mephedrone, normephedrone, 4-methylephedrine, and 4-methylpseudoephedrine in patient urines in relation to mephedrone concentration



5.3.4.2 Qualitative Identification of Other Metabolites from LC-HRMS Analysis

Pozo *et al.* (2015) identified 10 metabolites of mephedrone in urine collected 4 h post-ingestion of mephedrone (oral, 400 mg) from two male volunteers. For identification of metabolites that had no available reference material, the chromatographic data were filtered for the theoretical accurate precursor ion *m/z* and peak identity confirmed through the presence of product ions (Table 5.7). Metabolites identified in the patient urine samples are summarised in Table 5.8, and example chromatograms shown in Figure 5.7. No definitive chromatographic peaks were identified in patient samples which corresponded to the three glucuronide metabolites. As these metabolites are more polar, it is possible that they were not retained on the analytical column.

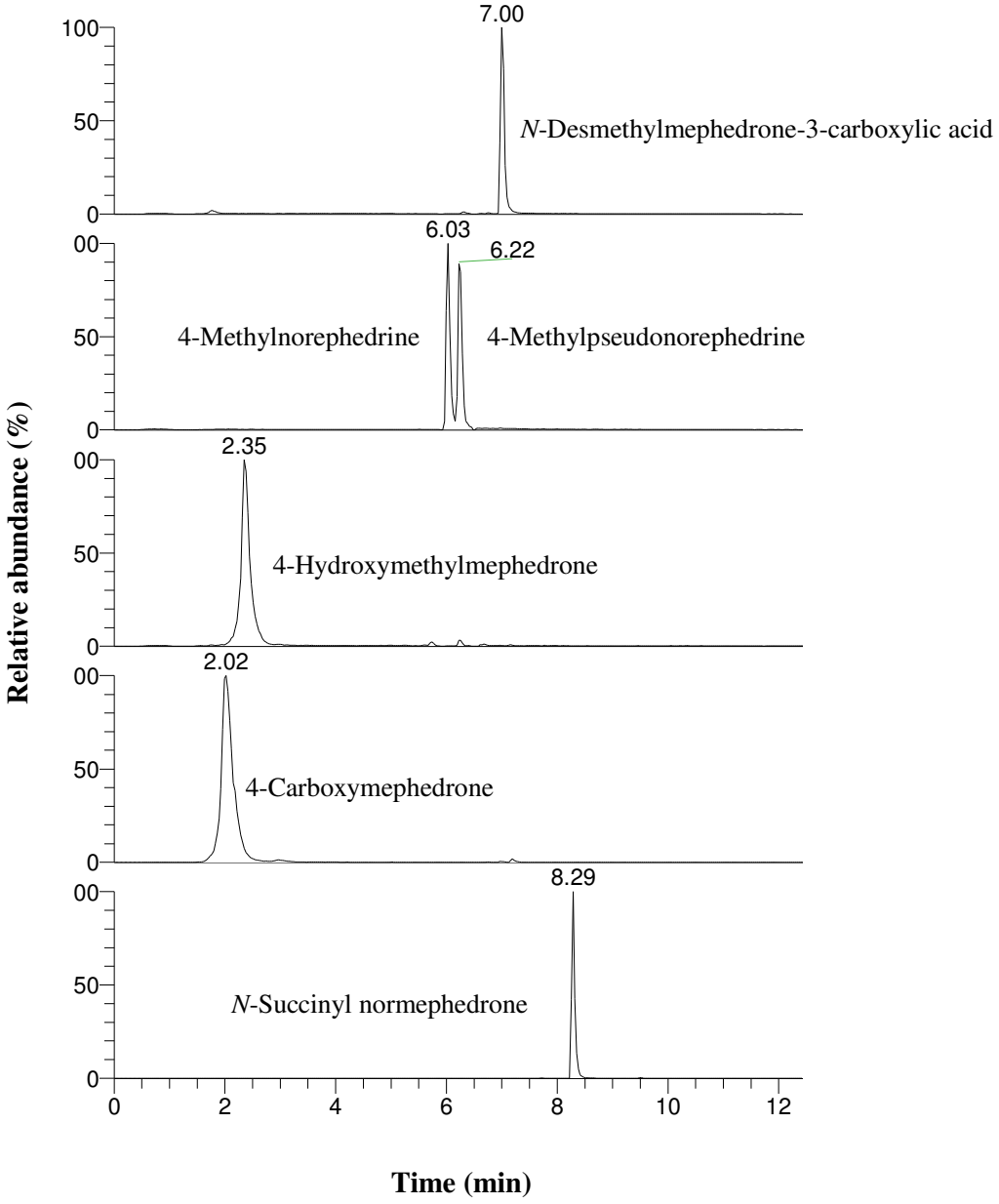
Table 5.7 – Accurate precursor and product ion *m/z* for selected urinary metabolites of mephedrone ascertained by Pozo *et al.* (2015)

Metabolite	**Observed precursor ion *m/z***	**Observed product ions *m/z***
Phase I		
N-Desmethylmephedrone-3-carboxylic acid	194.0823	119.0497 91.0547 138.0666
4-Methylnorephedrine/ 4-Methylpseudonorephedrine	166.1231	131.0861 148.1122 91.0548
4-Hydroxymethylmephedrone	194.1178	158.0961 146.0962 131.0728
4-Carboxymephedrone	208.0973	131.0735 146.0968 172.0762
Phase II		
N-Succinyl normephedrone	264.1234	146.0971 126.0555 119.0860
Hydroxymephedrone glucuronide	370.1509	194.1176 119.0497 160.1121
4-Carboxymephedrone glucuronide	384.1302	208.0982 172.0763 146.0968
Hydroxylnormephedrone glucuronide	356.1346	180.1028 162.0919 119.0478

Table 5.8 – Summary data: Qualitative identification of urinary metabolites of mephedrone in patient samples

	N	Mean (range) peak area	Mean RT (min)	Theoretical *m/z*	Mean measured *m/z*	Mass accuracy (ppm)
4-Hydroxymethylmephedrone	25	$2.0\ e^{9}$ ($7.8\ e^{6}$-$2.1\ e^{10}$)	2.26	194.1176	194.1173	-1.34
N-Succinyl normephedrone	25	$1.1\ e^{9}$ ($8.4\ e^{6}$-$3.7\ e^{9}$)	8.29	264.1230	264.1225	-1.71
4-Methylnorpseudoephedrine	25	$9.3\ e^{8}$ ($1.1\ e^{7}$-$3.5\ e^{9}$)	6.23	166.1226	166.1225	-0.53
4-Methylnorephedrine	24	$1.1\ e^{9}$ ($3.6\ e^{7}$-$3.3\ e^{9}$)	6.03	166.1226	166.1225	-0.65
N-Demethylmephedrone-3-carboxylic acid	22	$1.3\ e^{9}$ ($9.4\ e^{7}$-$7.5\ e^{9}$)	7.01	194.0812	194.0810	-0.89
4-Carboxymephedrone	16	$4.5\ e^{9}$ ($4.4\ e^{7}$-$2.7\ e^{10}$)	2.07	208.0968	208.0965	-1.53

Figure 5.7 – Extracted ion chromatograms to show selected mephedrone metabolites identified in a patient urine sample (mephedrone concentration 271 mg/L)



5.3.4.3 Analysis of Patient Samples by CEDIA

The measured amfetamine-group CEDIA concentration in patient samples is summarised in Table 5.9. The median amfetamine-group concentration was significantly lower in samples containing mephedrone in the absence of other amfetamines than in samples where other amfetamines (amfetamine, metamfetamine, MDMA) were present (Mann-Whitney U test, p = <0.001 two-tailed). The majority of samples (90 %) in which only mephedrone was detected had negative CEDIA results. The mephedrone concentration (measured by LC-HRMS, Section 5.3.4.1) was not significantly different between samples where other amfetamines were present and those where only mephedrone was present (Mann-Whitney U test, p = 0.37 two-tailed).

Table 5.9 – Summary of amfetamine-group CEDIA results in patient urine samples where mephedrone was detected by LC-HRMS (>0.01 mg/L), and categorisation according to whether other amfetamines were detected or not

*amfetamine, metamfetamine, or MDMA detected in addition to mephedrone

Patient samples where mephedrone was detected	N	No. of samples screened 'positive' by CEDIA	Median (range) amfetamine-group CEDIA concentration (mg/L)	Median (range) mephedrone concentration (mg/L)
All	25	15	1.14 (0.01-235)	10.5 (0.05-374)
Other amfetamines detected*	15	14	2.46 (0.10-235)	13.2 (0.05-271)
No other amfetamines detected	10	1	0.19 (0.01-3.04)	6.80 (0.08-374)

5.4 Conclusions

Collection of full scan data offers the possibility to analyse the data retrospectively. However, the process is not straight-forward and has limitations. At the time of the original sample analysis an appropriate internal standard may not have been included, thus analyte concentrations ascertained at a later date can only be estimations as the internal standard may not compensate appropriately for matrix effects. In addition, analyte concentrations may be above the linear range of the assay meaning that sample dilution would have been necessary for accurate results. In this case the sample should be re-analysed, but depending on how much time has passed since the original analysis the sample may have been discarded or the analyte may have degraded. For many NPS no stability data exist making it difficult to interpret results.

Another limitation is that reference materials are still required for confirmation of an analyte. Even in cases where a reference material is available for the compound of interest, co-elution of an isobaric compound cannot be excluded. Identification of specific MS^2 product ions may help minimise incorrect interpretation of results. For positional isomers, specific MS^2 product ions may be difficult to identify. In this study, reference solutions of 2-MMC and 3-MMC (positional isomers of mephedrone) could not be obtained. It therefore cannot be ruled out that measurement may be of these compounds as opposed to mephedrone itself in the patient samples. 2-MMC and 3-MMC were both identified in European drug seizures in 2014, with a greater quantity of 3-MMC seized than mephedrone itself (388 kg v. 222 kg) (EMCDDA, 2016).

Despite this, data gleaned from retrospective analyses are of value and can be used to guide future assay development, e.g. to gain information on appropriate calibration ranges and putative metabolites. This work confirms that normephedrone, 4-methylephedrine, and 4-methylpseudoephedrine are urinary metabolites of mephedrone. In addition, five of the reported urinary metabolites identified by Pozo *et al.* (2015) were qualitatively identified in the patient urines studied. To date, 4-methylpseudoephedrine and 4-methylpseudonorephedrine have not been reported as metabolites of mephedrone. This may be a result of previously published analytical methods not achieving chromatographic resolution between 4-methylephedrine and 4-methylpseudoephedrine, and between 4-methylnorephedrine and 4-methylpseudo-norephedrine. The estimated concentrations for mephedrone and its metabolites in this study are considerably higher than those reported by Concheiro *et al.* (2015), and provide more information on expected urinary concentrations from individuals who take

mephedrone. It may be that the individuals in our study population took higher doses of mephedrone, or that the purity of mephedrone was higher, which could explain the higher observed urinary concentrations.

The low cross-reactivity of mephedrone and its metabolites in the CEDIA assay highlight that immunoassay methods may be inappropriate for detection of mephedrone, and potentially other beta-keto amfetamines (e.g. butylone, methylone). The majority of patient samples containing mephedrone in the absence of other amfetamine drugs had a CEDIA concentration <0.5 mg/L resulting in false-negative results. This emphasises the need to assay these novel substances using specific methodology such as LC-HRMS.

5.4.1 Further Work

Changing the internal standard to mephedrone-^{13}C-D_3 (now available from Alsachim) could be investigated to reduce interference from the isotopes of 4-methylephedrine and 4-methylpseudoephedrine. However, for qualitative purposes based on a cutoff concentration of 0.2 mg/L (EWDTS guidelines) the use of mephedrone-D_3 as an internal standard should not impact results. In addition the prevalence of mephedrone appears to have declined in the UK (Home Office, 2010-2016) meaning that there may be limited clinical benefit to justify further refinement of the mephedrone assay.

6 Audit of Clinical Samples Received for Urine Drug Screening

6.1 Introduction

Transferring the process of urine drug screening to a single-step procedure (i.e. screening and confirmation in one assay) is a relatively new approach. Much of the established procedure for urine drug screening, including reporting of results, is based on the two-step process typically using cutoff concentrations to assess if a sample is 'positive' or 'negative' for a drug or drug class. Whether this is still an appropriate approach, particularly in regard to reporting results, in light of the new technology being used and the range of NPS now available has not been investigated. Evaluation of the data resulting from routine analysis of urine samples using LC-HRMS (Chapter 2) may give additional information that can be used to aid the clinical interpretation of results.

Ideally, knowledge of the clinical circumstances and the reason behind requesting a urine drug screen are required to enable bespoke interpretation of results. However, many of the samples received by the laboratory lack any clinical information whatsoever hence the results cannot be put in context. Be this as it may, most clinical samples received for urine drug screening are from drug and alcohol rehabilitation services, hence it can be assumed that the samples have been collected to ascertain whether an individual is compliant with prescribed medication, e.g. with methadone, and/or whether they are misusing other drugs.

6.1.1 Aims

The results and patient demographics from samples submitted for urine drug screening will be evaluated. The analytical interpretation of results will focus on the identification of sample adulteration, the ability to distinguish the use of heroin from pharmaceutical diamorphine, and ascertaining the prevalence of heroin use in individuals receiving OST in a user population. In addition, the impact of reporting results based on the limit of analytical detection as opposed to cutoff concentrations will be studied.

6.2 Methods

6.2.1 Patient Samples

The results from the analysis of samples submitted for urine drug screening from UK patients to King's College Hospital (November 2015-October 2016 inclusive) were audited. Information requested at the time of sample submission included requestor details, time and date of sample, patient date of birth, sex, tests required and prescribed medication (e.g. methadone, buprenorphine, benzodiazepines).

6.2.2 Analytical Methods

Most analytes were measured using LC-HRMS (Chapter 2). The use of benzodiazepines, cannabis, and barbiturates was detected using immunoassay (CEDIA, ThermoFisher Scientific). Ethanol was measured using an enzymatic assay (DRI, ThermoFisher Scientific). Creatinine was measured to monitor sample integrity (Section 1.2.3.1) using the Jaffe reaction (Creatinine Detect, ThermoFisher Scientific). Barbiturate and ethanol assays were not performed routinely as part of a urine drug screen, but were available on request.

For audit purposes, two sets of data were generated based on a) analytical detection limits (LoD), and b) cutoff concentrations (Section 2.3.4). For clinical purposes analytes were reported as either 'positive' or 'negative', based on the cutoff concentrations for LC-HRMS (Table 2.6) and according to immunoassay kit manufacturer instructions (benzodiazepine group and barbiturate group 300 µg/L, cannabis 50 µg/L). Results based on the LoD are reported as either 'detected' or 'not detected'. For immunoassay/enzymatic assay the manufacturer stated LoD was used (barbiturates 36.3 µg/L, benzodiazepines 8.3 µg/L, cannabis 11.8 µg/L, and ethanol 10 mg/100 mL) and for LC-HRMS the LoD ascertained during method validation was used (Table 2.13).

Drug classes were defined as: amfetamines, benzodiazepines, buprenorphine, cannabis, cocaine, ketamine, methadone, opioids, and tramadol.

6.2.3 Statistical Analysis

The semi-quantitative results were used for statistical analyses. Statistical analyses were performed using Microsoft Excel 2010 (version 14.0.7166.5000) and Analyse-It (version 2.3). The Shapiro-Wilk test was used to test normality of data. For non-parametric data, the Kruskal Wallis H test or the Mann-Whitney U test was used to compare data.

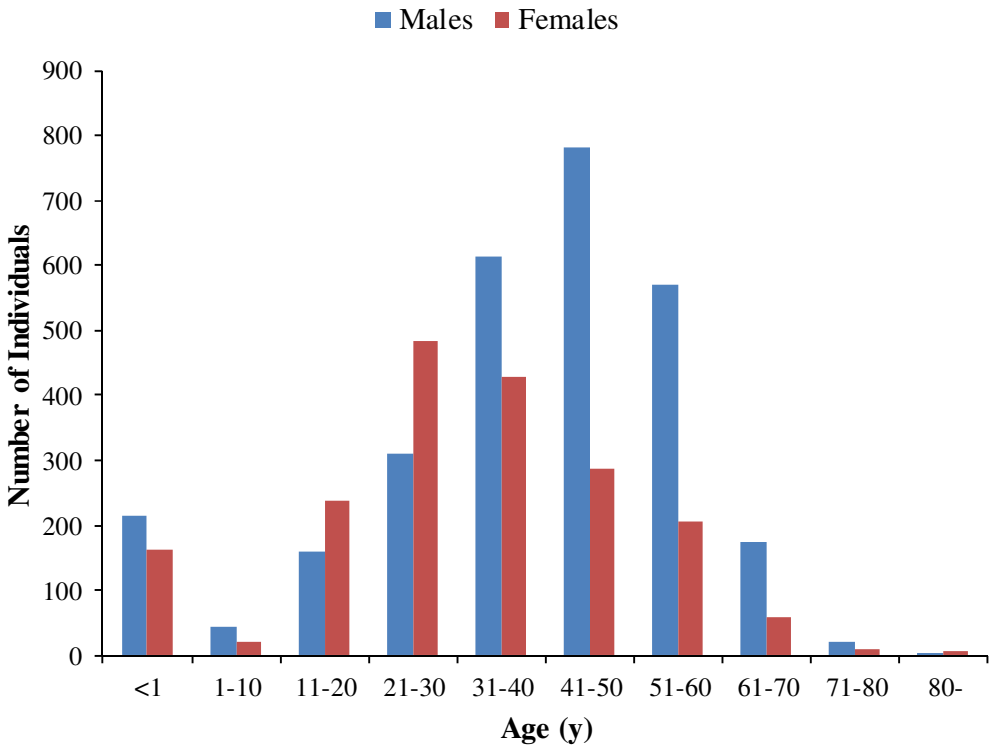
6.3 General Findings

There were 8,506 samples from 4,800 patients. Of these, 257 had barbiturate and 248 had ethanol tests requested in addition to the routine screen (3 % of all samples received in both cases).

6.3.1 Demographics

There were 2,899 (60.4 %) males aged at the time of first sampling (median, range) 44 years (0-89), and 1,901 (39.6 %) females aged at the time of sampling (median, range) 35 years (0-84) (patient age not known for 6 samples, all males). The age and sex distribution of the individuals from whom urine samples were received is summarised in Figure 6.1. The number of samples per patient was as follows: 1 (3,601 patients), 2-5 (948), 6-10 (193), 11+ (58). The maximum number of sample received from one patient was 53.

Figure 6.1 – Age and gender distribution of individuals from whom urine samples were received



Most samples were received from locations within the south-east of England, with the majority of requestors located in the boroughs of Southwark and Lambeth in London. Most requestors were part of drug and addiction services (42 %). Other requests came via external biochemistry laboratories (32 %), general practitioners (18 %), from within King's College Hospital (7 %), and from mental health services (2 %).

6.3.2 Drug prevalence

No drugs were detected in 1,976 samples (23.3 %). Most of these samples were from external biochemistry laboratories (65 %). The remaining samples were from within King's College Hospital (15 %), from drug and addiction services (9 %), and from general practitioners (4 %).

Where drugs were detected, the number of drug classes screened as positive in single urine sample ranged from 1 to 7 (Table 6.1).

Table 6.1 – Number of drug classes positive in each urine sample

Drug classes positive	Number of samples	% samples
1	2,092	24.6
2	1,824	21.4
3	1,607	18.9
4	800	9.4
5	189	2.2
6	17	0.2
7	1	0.01

The most commonly detected drug was methadone (38 % samples with methadone and/or EDDP detected), followed by morphine (32 % samples). Detection was relatively infrequent for amfetamines (2 %) (Table 6.2). In samples where a barbiturate test was requested, only 2 samples (0.8 %) gave positive results. In both cases, administration of barbiturates was detailed on the sample request form.

Table 6.2 – Drugs screened as 'positive' in samples analysed at King's College Hospital November 2015-October 2016 inclusive (N = 8,506)

* Specific drugs within a drug class are italicised, a sample was defined 'positive' for a drug class if at least one of the listed drugs was positive

41 samples were positive for amfetamine and metamfetamine, with the presence of amfetamine likely a result of metamfetamine metabolism

Drug class/Drug	No. of 'positive' samples based on defined cutoffs	% samples positive
Amfetamines*	173	2.0
Amfetamine#	*125*	*1.5*
Metamfetamine	*51*	*0.6*
MDMA	*40*	*0.5*
Mephedrone	*10*	*0.1*
Benzodiazepines	1,310	15.4
Buprenorphine*	1,448	17.0
Buprenorphine	*357*	*4.2*
Norbuprenorphine	*1,286*	*15.1*
Buprenorphine glucuronide	*1,295*	*15.2*
Norbuprenorphine glucuronide	*1,348*	*15.8*
Cannabis	2,508	29.5
Cocaine (as benzoylecgonine)	2,708	31.8
Methadone*	3,230	38.0
Methadone	*3,102*	*36.5*
EDDP	*3,113*	*36.6*
Opioids*	3,052	35.9
Total Morphine	*2,729*	*32.1*
Total Codeine	*2,262*	*26.6*
Total Dihydrocodeine	*223*	*2.6*
Pholcodine	*17*	*0.2*
6-AM	*1,358*	*16.0*
Ketamine	45	0.5
Tramadol	265	3.1

6.4 Sample Adulteration

6.4.1 Background

Sample adulteration is one of the problems encountered in urine drug screening (Section 1.2.3.1). Individuals may directly add prescribed medication (e.g. methadone, buprenorphine) to their urine sample in an attempt to appear adherent. These cases may be distinguished analytically through detection of either a high concentration of parent drug in relation to its metabolites, or the absence of metabolites.

Buprenorphine immunoassays cannot differentiate between buprenorphine and metabolites, thus adulteration cannot be detected using this methodology (Belsey *et al.*, 2014). Specific immunoassays exist for both methadone and EDDP meaning that adulteration cases can be detected, but this requires two separate assays which increases laboratory expenditure (Preston *et al.*, 2003). Most clinical laboratories using immunoassay will solely use the EDDP assay, which will provide information on methadone adherence; however, cases of adulteration will be missed. LC-MS methods are capable of detecting metabolites in addition to the parent drugs, and are well suited to identifying cases of sample adulteration.

As methadone is a liquid, direct addition of the medication to urine will result in sample dilution. Dilution of a sample may be ascertained through creatinine measurement. Urine samples may be classified as dilute, and very dilute, when creatinine is <1.8 and <0.2 mmol/L, respectively (SAMHSA, 2012).

6.4.2 Results and Discussion

6.4.2.1 Direct Addition of Methadone and Buprenorphine

Based on reporting according to cutoff concentrations, 117 samples were positive for methadone and negative for EDDP. The median (range) methadone concentration in these samples was 9.9 (0.3-773) mg/L. EDDP was detected below the cutoff (0.25 mg/L) in 50 of these samples. The median (range) EDDP concentration was 0.15 (0.02-0.24) mg/L. Where EDDP was detected, the methadone-to-EDDP ratio was calculated and compared with the methadone concentration (Figure 6.2). A methadone-to-EDDP ratio >50 appears suggestive of sample adulteration. Cases of adulteration were identified by samples having a high methadone concentration in combination with either a) no EDDP detected, or b) a methadone-to-EDDP ratio >50.

In 1,500 patient samples where buprenorphine and/or buprenorphine metabolites were detected, the proportions of each species (data converted to µmol/L) present were calculated. Buprenorphine comprised <15 % of the total species in urine in 93 % samples, with norbuprenorphine glucuronide usually being the predominant species present. Cases of adulteration were clearly identified by the disproportionately high buprenorphine concentration (Figure 6.3). Eighteen samples were positive for buprenorphine with no metabolites detected. The median (range) buprenorphine concentration in these samples was 47.1 (5.7-16,000) µg/L. Seven samples had a buprenorphine concentration >1,000 µg/L indicating adulteration. In samples where buprenorphine and at least one metabolite were detected (N = 535), the buprenorphine concentration and the buprenorphine-to-metabolites ratio was compared (Figure 6.4). Data suggest sample adulteration when urinary buprenorphine concentration is >1,000 µg/L and the buprenorphine-to-metabolites ratio is >1.

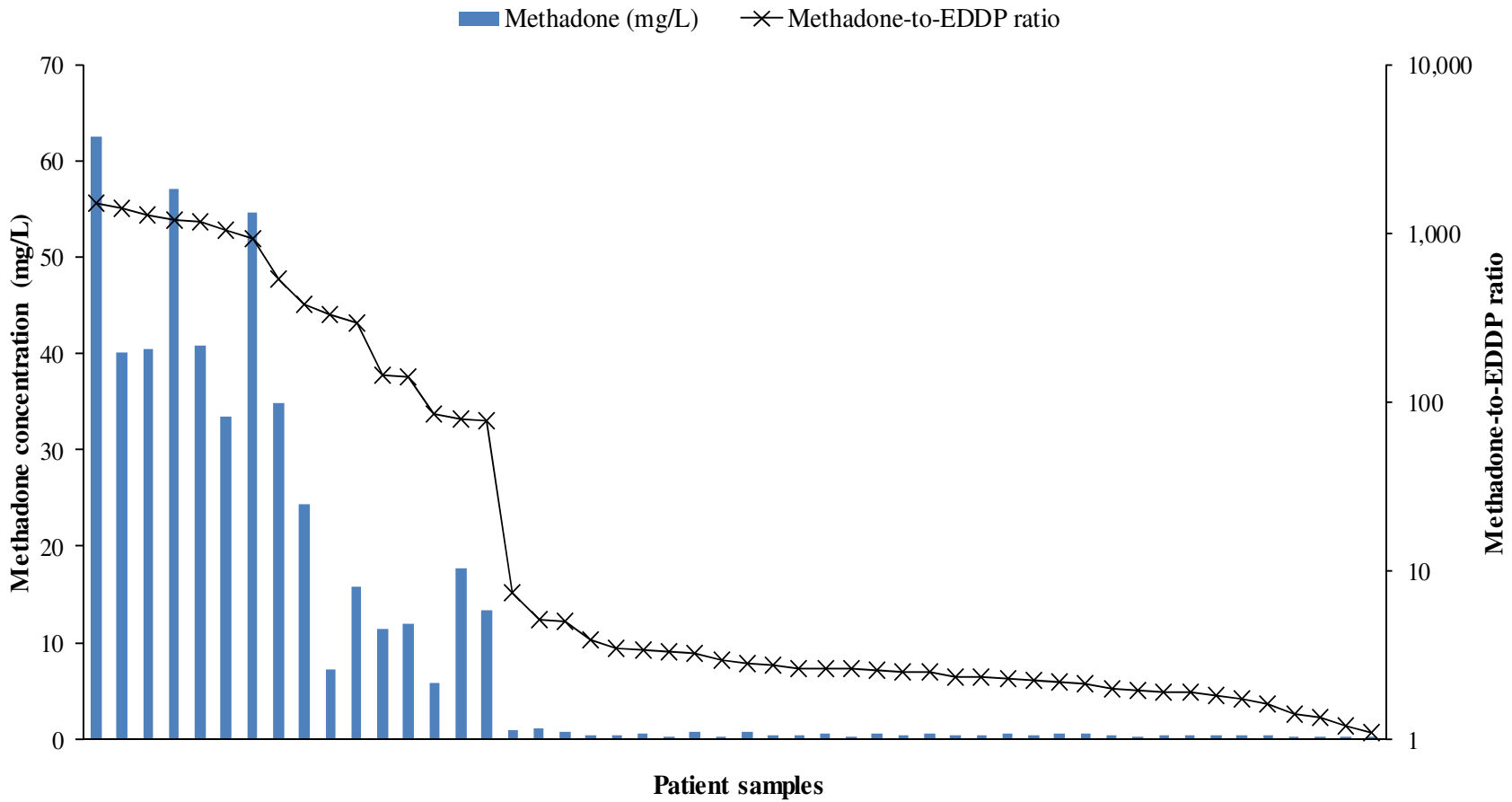
Overall, 153 samples from 91 patients were identified as adulterated with either methadone (83 samples from 57 patients) or buprenorphine (70 samples from 34 patients) (Table 6.3).

6.4.2.2 Creatinine

Thirteen samples (0.2 % total samples) from 9 individuals (6 males, 3 females) had no creatinine detected (<0.07 mmol/L). Of these, 11 samples were adulterated through addition of methadone, and 1 sample through addition of buprenorphine. The remaining sample had no buprenorphine or methadone detected, but was observed to have a high 6-AM concentration (~34 mg/L) in the absence of morphine and codeine suggesting possible addition of diamorphine or heroin (Section 6.5).

Twenty-two samples (0.3 %) from 15 individuals (12 males, 3 females) were classed as ‘very dilute’, based upon a creatinine concentration <0.2 mmol/L. Of these, 9 samples were adulterated with methadone, and 3 samples were adulterated with buprenorphine. The remaining 10 (45 %) samples had no clear evidence of sample adulteration. 5 of these samples had no drugs detected. It is possible that these urine samples were purposefully diluted to obtain negative results, either by direct addition of a liquid or through drinking copious amounts of fluid.

Figure 6.2 – Comparison of the methadone concentration and methadone-to-EDDP ratio in methadone positive patient urine samples where EDDP was detected below the cutoff concentration (<0.25 mg/L) (N = 50)



<u>Figure 6.3</u> – Percentage of buprenorphine and metabolites (µmol/L) in a subset of patient urine samples (N = 100, selected samples were the first 100 positive buprenorphine samples from the audit data)

Key: Buprenorphine (blue), norbuprenorphine (red), buprenorphine glucuronide (green), and norbuprenorphine glucuronide (purple)

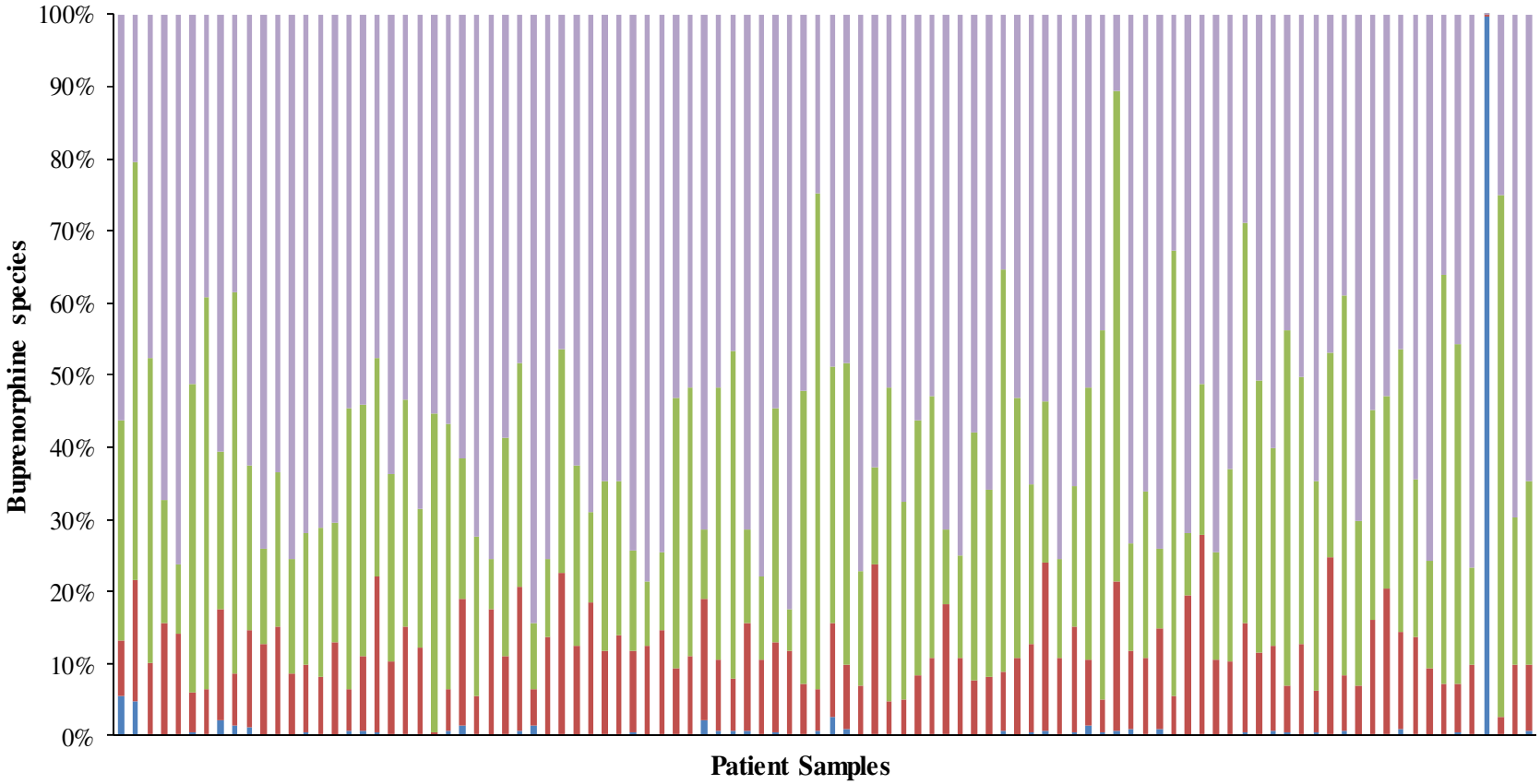


Figure 6.4 – Comparison of the buprenorphine concentration and buprenorphine-to-metabolites ratio in patient urine samples where buprenorphine and at least one metabolite was detected (N = 535)

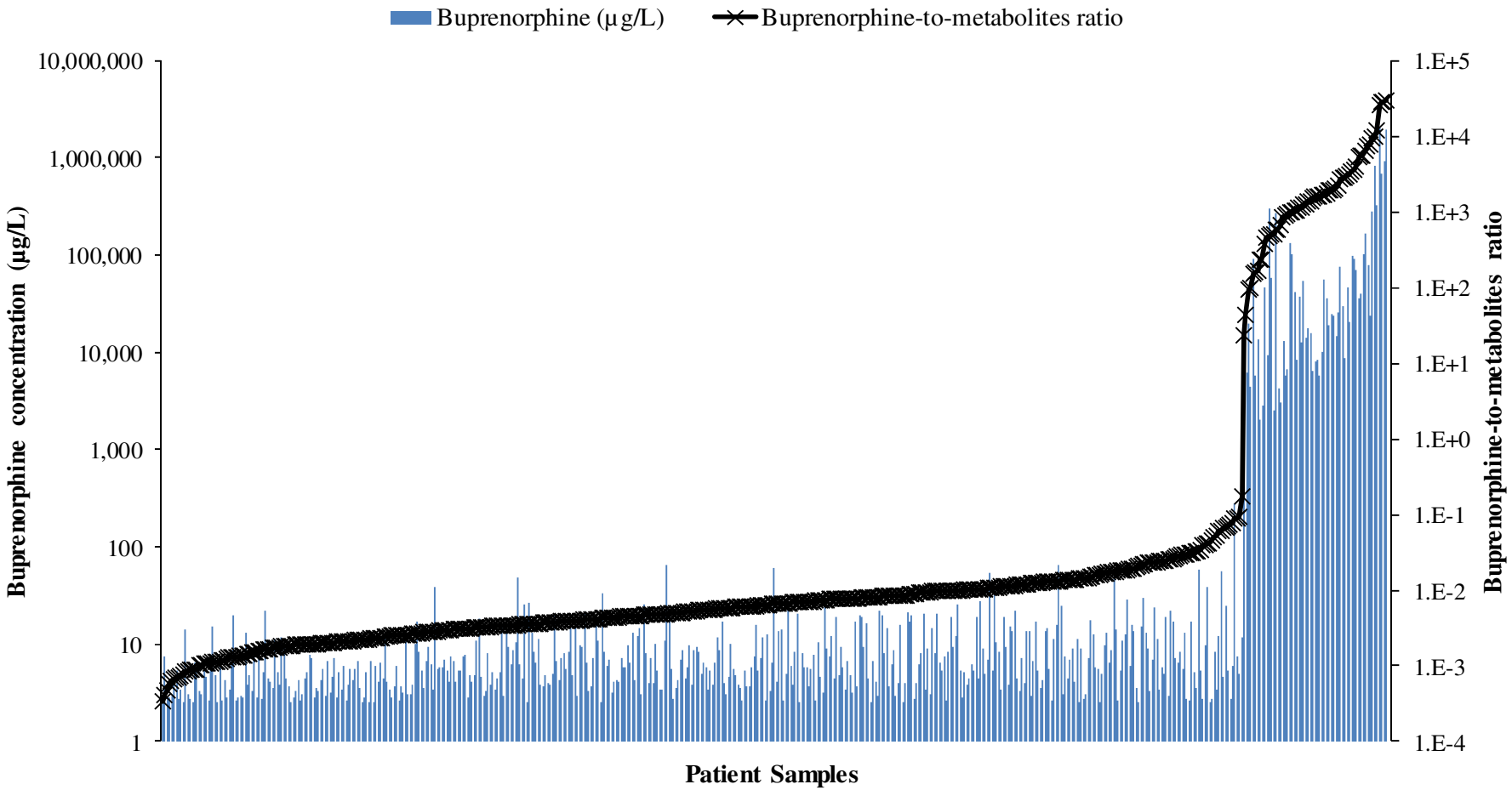


Table 6.3 – Summary of the analytical findings in patient urine samples that were adulterated by direct addition of methadone or buprenorphine

ND – Not detected (<0.07 mmol/L)

		Creatinine		
		ND	**<0.2 mmol/L**	**>0.2 mmol/L**
Methadone adulterations	High methadone concentration, no EDDP detected	9	8	50
	Methadone-to-EDDP ratio >50	2	1	13
Buprenorphine adulterations	Buprenorphine >1000 µg/L, no metabolites detected	0	1	6
	Buprenorphine-to-metabolites ratio >1	1	2	60

6.4.3 Conclusions

1.8 % of all samples received were identified as adulterated through direct addition of methadone or buprenorphine. Identification of these cases could not solely be based upon a positive result for the parent drug and negative result for metabolites, particularly for buprenorphine. Calculation of the parent drug-to-metabolite ratio provided useful additional information in many cases.

6.5 Detection of 6-AM in conjunction with low total morphine concentration

6.5.1 Background

6-AM is the hydrolytic product of diamorphine, and is present in heroin and in pharmaceutical diamorphine (McLachlan-Troup *et al.*, 2001). The presence of diamorphine in urine is unexpected due to its rapid hydrolysis *in vivo*. It has been suggested that high concentrations of diamorphine and/or 6-AM may inhibit the enzyme responsible for glucuronidation of morphine (UGT 2B7), however free morphine would be expected in urine if this were the case (von Euler *et al.*, 2003).

The presence of 6-AM in the absence of morphine and/or morphine glucuronide has been reported (Andersson *et al.*, 2015; Beck and Böttcher, 2006; Glass *et al.*, 1997; von Euler *et al.*, 2003). Several reasons for this atypical pattern have been proposed including; sample timing (i.e. heroin intake was immediately prior to sampling), a result of a metabolic defect (e.g. lack of esterases that metabolise 6-AM to morphine), and inhibition of carboxylesterase from a substance co-ingested with heroin (either an unknown constituent of the heroin preparation, or a different drug). Genetic polymorphism in the enzymes involved in diamorphine/6-AM metabolism is deemed unlikely to cause this atypical pattern as studies have demonstrated the same individual can produce the atypical and 'normal' excretion pattern on different occasions (Andersson *et al.*, 2015; von Euler *et al.*, 2003). An alternative explanation is that diamorphine and 6-AM may be present as a result of direct addition of heroin to a urine sample.

6.5.2 Results and Discussion

From the entire data set, 49 samples from 35 individuals had positive 6-AM results with negative total morphine and total codeine results (i.e. less than the cutoff concentration, 300 µg/L). 22 of these samples had no morphine or codeine (or their glucuronides) detected. A high proportion of these samples (57 %) were adulterated through direct addition of methadone (45 %) or buprenorphine (12 %). Diamorphine (*m/z* 370.1649) was qualitatively identified in 48 samples through retrospective analysis.

Twenty-seven of the individuals had submitted at least one other urine sample during the study period. The majority of these individuals (81 %) showed a normal excretion pattern in urine samples collected at a different time. This supports the finding of Andersson *et al.* (2015) and von Euler *et al.* (2003), suggesting that the observed results are unlikely to be a result of genetic polymorphism.

6.5.3 Conclusions

The cause of this different excretion pattern in selected urine samples could not be established. Adulteration by direct addition of heroin to the urine sample cannot be excluded, particularly considering that the majority of the samples with the atypical pattern had been adulterated with either methadone, or buprenorphine.

6.6 Differentiation between pharmaceutical diamorphine and heroin use

6.6.1 Background

Heroin-assisted treatment (HAT) refers to the prescribing of diamorphine to opioid-dependent individuals. There is in such cases a clinical need to differentiate between the administration of prescribed diamorphine and heroin use. A number of alkaloids in addition to morphine are produced by *Papaver somniferum* (opium poppy), including codeine, noscapine, papaverine, meconin, and thebaine. The alkaloids present in heroin vary, and depend on the climatic conditions the poppy was grown under and the extraction method used to concentrate morphine (Bogusz *et al.*, 2001). These co-extracted alkaloids and their acetylated derivatives are absent in pharmaceutical diamorphine, and offer potential markers of concurrent heroin use in patients prescribed diamorphine (McLachlan-Troup *et al.*, 2001).

To date, 6-acetylcodeine has been identified as the most specific marker of illicit heroin use. However, due to rapid conversion to codeine *in vivo,* 6-acetylcodeine has a short urinary detection window (<8 h) which limits its clinical utility (Bogusz *et al.*, 2001; Brenneisen *et al.*, 2002; O'Neal and Poklis, 1997, 1998). In addition, co-administration of codeine and pharmaceutical diamorphine may produce 6-acetylcodeine *in vivo* (Brenneisen *et al.*, 2002).

Other opium alkaloids (e.g. papaverine, noscapine) may be present in urine samples as a result of the ingestion of poppy seeds, and thus cannot be used as specific markers of heroin use. Noscapine and papaverine may also result from administration of medicines. Noscapine is a component of some OTC cough suppressants, and papaverine may be used to treat impotence and cardiovascular disease (Paterson *et al.*, 2005). Papaverine has also been detected in individuals administered atracurium, a neuromuscular relaxant administered to facilitate tracheal intubation, as a result of dehydrogenation of laudanosine (the major metabolite of atracurium) (Seetohul *et al.*, 2013). Both noscapine and papaverine are extensively metabolised. Papaverine has a short plasma half-life (0.8-1.5 h) and is metabolised mainly to 6-hydroxypapaverine and other hydroxylated metabolites, all of which may be glucuronidated. Noscapine has a longer plasma half-life (4.5 h) and is more likely than papaverine to be detected as unchanged drug in urine samples. The major urinary metabolites of noscapine are meconin and cotarnine.

A novel marker of illicit heroin use has recently been reported, acetylated-thebaine-4-metabolite glucuronide (ATM4G) (Chen *et al.*, 2014). ATM4G is not formed metabolically after poppy seed ingestion, and therefore may enable detection of heroin consumption (Chen *et al.*, 2014; Maas *et al.*, 2017). Currently no reference material is commercially available for this compound; hence quantitative measurement is not possible.

An alternative approach to identifying heroin use through calculation of the morphine to codeine ratio in either blood or urine has been proposed. Studies have suggested that a ratio greater than 1 is suggestive of heroin use (Ceder and Jones, 2001; Jones *et al.*, 2008; Konstantinova *et al.*, 2012).

6.6.2 Results and Discussion

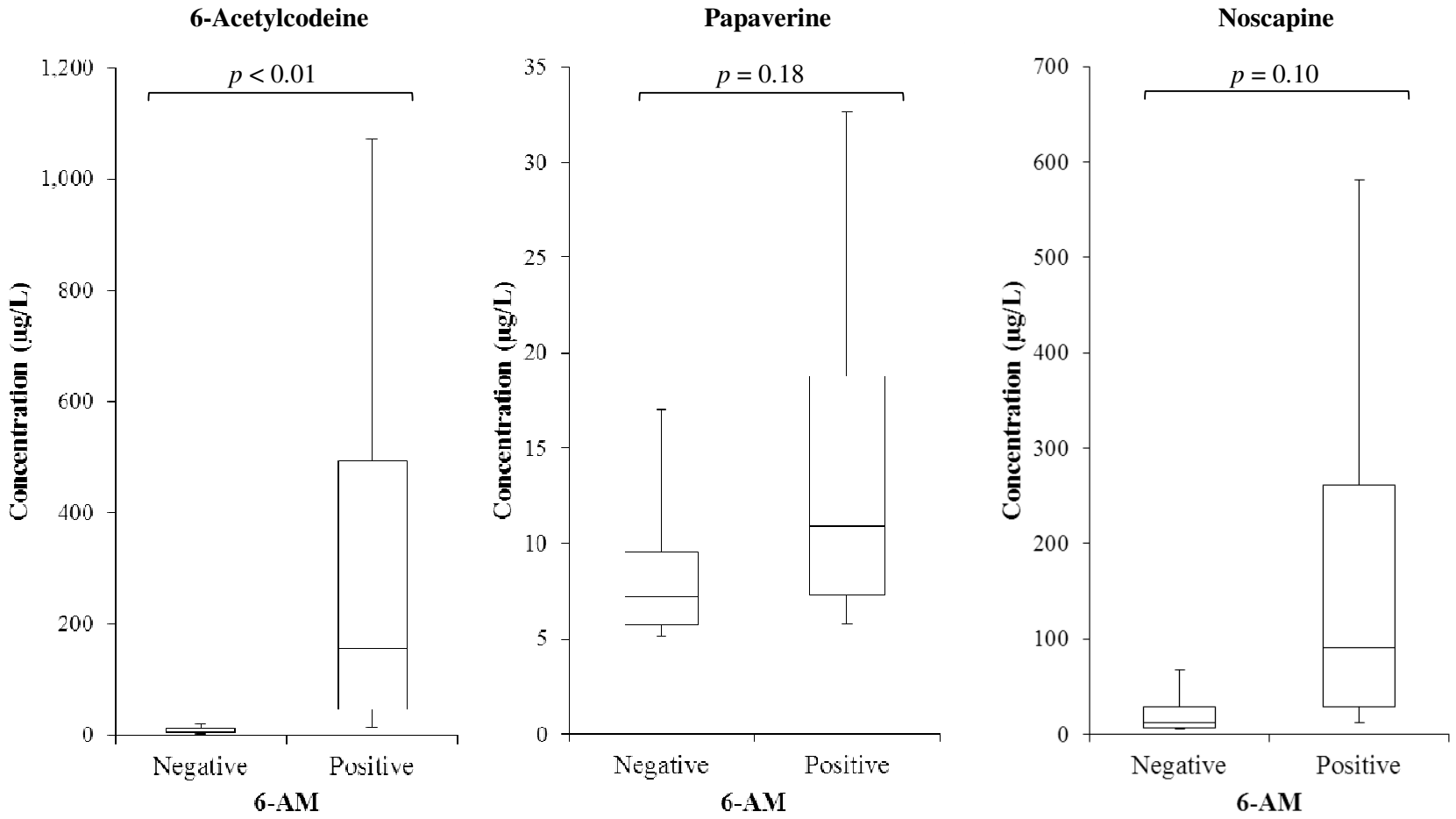
6.6.2.1 Heroin Markers

Papaverine was typically detected at lower concentration and less frequently than 6-acetylcodeine and noscapine (Table 6.4). 6-AM was present in most samples containing these markers, confirming the use of heroin. To assess the suitability of each marker for identifying the use of heroin, the concentration of the marker in 6-AM positive samples was compared to that in 6-AM negative samples (Figure 6.5). 6-Acetylcodeine was the only marker observed to have a significantly higher median concentration in 6-AM positive samples (p <0.01). If 6-acetylcodeine is used as a marker of heroin use, an additional 43 samples where 6-AM was either detected below the cutoff concentration (40 %) or not detected (60 %) could be identified. Identification of heroin use based solely on detection of noscapine or papaverine is unreliable as a significantly higher concentration in 6-AM positive samples was not observed.

Table 6.4 – Prevalence of 6-acetylcodeine, noscapine, and papaverine in urine samples

Marker	N	% samples with 6-AM detected	Concentration (µg/L)			
			Mean	Median	Min	Max
6-Acetylcodeine	1,077	98	412	145	3	6,600
Noscapine	1,668	77	271	49	5	135,000
Papaverine	467	81	186	10	5	41,200

Figure 6.5 – Box and whisker plots (median, 25th-75th percentiles, whiskers 10th and 90th percentiles) to show the difference in heroin marker concentration in patient urine samples where 6-AM was positive versus those samples where 6-AM was negative (6-AM cutoff = 10 µg/L)



6.6.2.2 Morphine-to-codeine Ratio

2,258 samples contained morphine and codeine, with 1,269 (56 %) positive for 6-AM (>10 µg/L). For samples where 6-AM was positive, a morphine-to-codeine ratio >1 was observed in 99 % of the cases. There was a statistically significant difference in the morphine-to-codeine ratio between 6-AM positive samples, 6-AM negative samples with heroin markers detected, and 6-AM negative samples without heroin markers detected ($\chi^2(2) = 445.05$, $p < 0.0001$). The median morphine-to-codeine ratio was lower in samples where no heroin markers were detected as opposed to samples where heroin markers were detected in both the presence and absence of 6-AM (2.1 compared to 8.8 and 8.0, respectively) (Figure 6.6).

Fifteen samples had a very high morphine-to-codeine ratio (>50) in the absence of 6-AM and heroin markers (Figure 6.7). Prescription of morphine (either oramorph or morphine sulfate) was stated on the request form for the majority (60 %) of these samples which would account for a high morphine concentration in the absence of 6-AM. Codeine present in these samples may have arisen from co-ingestion of codeine; however neither the use of heroin, nor ingestion of poppy seeds can be excluded. A higher than expected morphine-to-codeine ratio may be also seen in CYP2D6 ultra-rapid metabolisers after codeine administration, which could be misinterpreted as heroin intake (He *et al.*, 2008).

Figure 6.6 – Box and whisker plots (median, 25th-75th percentiles, whiskers 10th and 90th percentiles) to show the difference in morphine-to-codeine ratio between patient urine samples where a) 6-AM was positive, b) 6-AM was negative, but heroin markers were detected, and c) 6-AM was negative and no heroin markers were detected

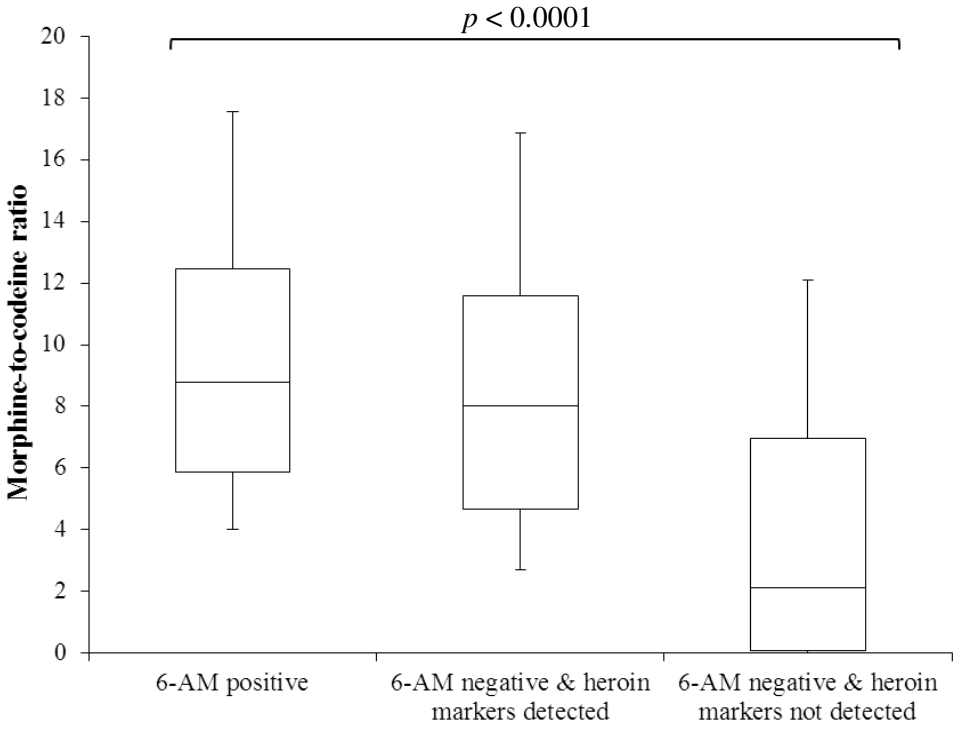
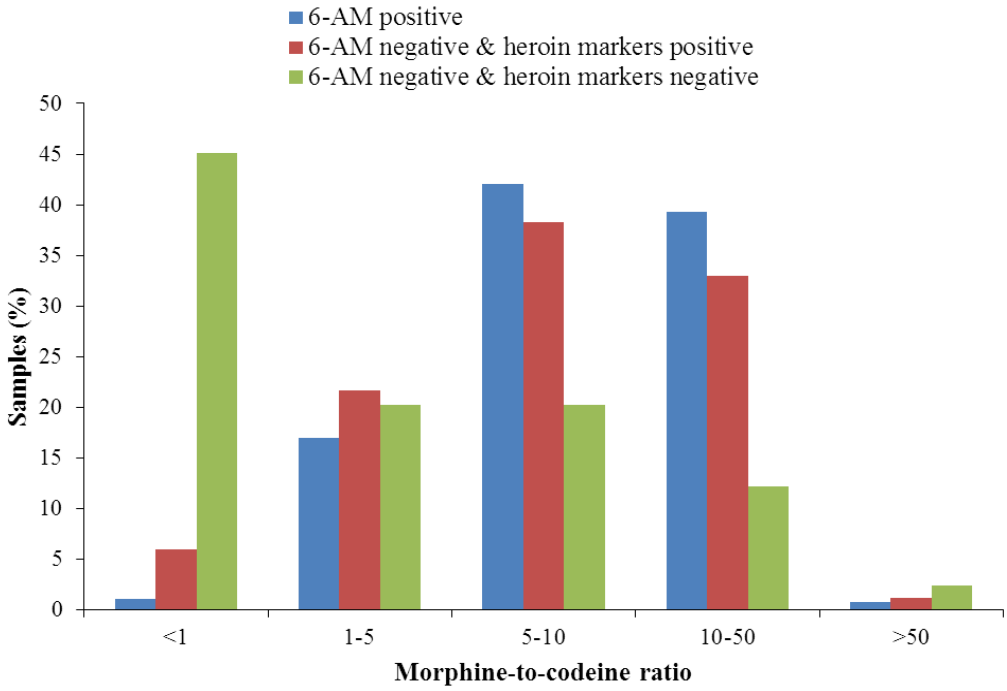


Figure 6.7 – The observed morphine-to-codeine ratio in patient urine samples according to whether 6-AM was positive or negative, and whether heroin markers were detected or not



6.6.3 Conclusions

Detection of 6-acetylcodeine, noscapine, and papaverine may be useful for differentiating the use of heroin and prescribed diamorphine. The detection of 6-acetylcodeine provides conclusive evidence that heroin as opposed to pharmaceutical diamorphine has been used. However, the detection window for 6-acetylcodeine in urine is short which limits its utility as a heroin marker. Data show 6-acetylcodeine is typically detected in samples where 6-AM is also detected. For identifying the use of heroin or diamorphine in the absence of 6-AM, the morphine-to-codeine ratio does offer interpretative value. However, this marker cannot be used alone as higher ratios may be a result of co-ingestion of morphine and codeine, or from poppy seed ingestion.

6.6.3.1 Further Work

Retrospective interrogation of data for the presence of ATM4G, particularly in relation to the presence of 6-AM, other heroin markers, and the morphine-to-codeine ratio, would be of value to assess its potential as an additional qualitative identifier of heroin use. However, due of the vast quantity of data the process of retrospective interrogation would take a long time to complete.

6.7 Heroin use in individuals receiving opioid substitution therapy

6.7.1 Background

In the UK, most individuals undergoing substitution therapy will be prescribed either methadone or buprenorphine. Both drugs are effective at reducing the use of heroin (Mattick *et al.*, 2009, 2014). However, a proportion of patients enrolled in substitution therapy will continue to misuse opiates. Limited data are available regarding the use of pharmaceutical diamorphine as OST, but preliminary results suggest there is a reduction in heroin use when compared to individuals receiving methadone (Ferri *et al.*, 2011).

It is unclear whether the prevalence of heroin use is different between individuals prescribed buprenorphine and methadone. Many studies report no significant difference in opiate misuse between individuals prescribed buprenorphine or methadone (Gerra *et al.*, 2004; Mattick *et al.*, 2002; Pani *et al.*, 2000). However, some studies have found that patients prescribed buprenorphine have a lower prevalence of heroin use when compared to those receiving methadone (Curcio *et al.*, 2011; Giacomuzzi *et al.*, 2003; Vigezzi *et al.*, 2006). Buprenorphine possesses stronger affinity for μ opioid receptors than many opioid agonists (e.g. diamorphine, morphine, methadone), which blocks the effects of heroin and thus may discourage use. At adequate doses methadone will also block the effects of additional opiates, however if doses are inadequate individuals may use heroin in order to prevent withdrawal symptoms (Vigezzi *et al.*, 2006).

6.7.2 Method

To ascertain the rate of concurrent heroin use amongst individuals receiving either methadone or buprenorphine treatment, patient urine samples which were positive for either methadone or buprenorphine and had evidence of recent heroin use were compared to the total number of samples positive for methadone and buprenorphine. Recent heroin use was identified in samples where 6-AM and at least one heroin marker were detected, and the morphine-to-codeine ratio was greater than 1. A chi-squared test was used to compare the two treatment groups with respect to urinalysis results.

Urine samples positive for methadone or buprenorphine were taken to be a result of prescribed medication. Adulterated samples were excluded from the analysis.

6.7.3 Results and Discussion

The proportion of individuals using heroin was significantly different between those prescribed methadone and those prescribed buprenorphine, 32 % and 16 % respectively (χ^2 (1, N = 1,540) = 40.78, p <0.001). Interpretation of these data is difficult due to the lack of clinical information. Differences may exist between the two sets of patients, which might account for the observed difference in heroin use. For example, a greater number of patients may be at the start of treatment in the methadone group and the correct dosage may not have been established, which could cause a higher rate of heroin use in these individuals.

The interpretation is also limited by the fact that no information on whether methadone or buprenorphine was prescribed was available. It therefore cannot be ruled out that the methadone or buprenorphine present in these samples was a result of non-prescribed use.

6.7.4 Conclusions

Overall, 27 % individuals receiving OST (either methadone or buprenorphine) had used heroin during the study period. This may be an underestimation of heroin use as some urine samples will not contain 6-AM if sample collection was more than 24 h after heroin administration. Despite this, the data show that continued heroin use is a problem amongst individuals receiving methadone and also those receiving buprenorphine.

6.8 Reporting using cutoff concentrations and limits of analytical detection

6.8.1 Background

The use of analyte or group cutoff concentrations largely originates from the use of immunoassays for drug screening where false positive results occur due to limitations with the methodology. Using selective methodology such as LC-HRMS for drug screening should minimise the occurrence of false positive results, and it may therefore be more appropriate to use the limit of analytical detection as opposed to a cutoff concentration to minimise the reporting of false negative results.

Analytical detection limits will likely be lower than assigned cutoff concentrations. As a result, the window of drug detection will be extended. In some cases this may be beneficial, for example in cases where drug exposure may have been a single event, e.g. DFSA, drug administration to children. However, for monitoring chronic drug use in individuals it may make clinical interpretation more difficult as recent drug use may be more difficult to distinguish from historic use.

6.8.2 Results and Discussion

Drugs were detected in a number of patient urine samples, but at a concentration below the cutoff concentration (Table 6.5). Amfetamines, particularly MDMA and mephedrone, were commonly detected at concentrations below the cutoff. It may be that these are cases of occasional drug use, and the urine sample was collected some days after drug administration.

Buprenorphine and its metabolites have the lowest assigned cutoff concentration of the analytes studied (5 µg/L). After therapeutic administration, the urinary concentration of free (unconjugated) buprenorphine is often less than 1 µg/L (Debrabandere *et al.*, 1991). It is therefore not surprising that the buprenorphine concentration was below the cutoff in 38 % samples where buprenorphine was detected. For buprenorphine metabolites, only a small proportion of samples had a metabolite detected below the cutoff. Buprenorphine metabolites are reported as a summed total (as buprenorphine equivalents, Section 2.3.4), and when calculated no samples had a total concentration below the cutoff. In this case, there would be little difference when basing the interpretation of results on the LoD as opposed to cutoff concentration.

Table 6.5 – The number of patient urine samples where drugs were detected below the cutoff concentration

* concentration based on the sum of parent drug and metabolite/s

Drug class/Drug	No. of samples detected	No. of positive samples	% samples below cutoff (from <16 y)
Amfetamines			
Amfetamine	198	125	37 (7)
Metamfetamine	62	51	18 (0)
MDMA	101	40	60 (0)
Mephedrone	27	10	63 (0)
Buprenorphine			
Buprenorphine	572	357	38 (0)
Norbuprenorphine	1,365	1,286	6 (1)
Buprenorphine glucuronide	1,322	1,295	2 (3)
Norbuprenorphine glucuronide	1,357	1,348	1 (0)
Cocaine (as benzoylecgonine)	3,420	2,708	21 (6)
Methadone			
Methadone	3,256	3,102	5 (9)
EDDP	3,302	3,113	6 (10)
Opioids			
Total Morphine	3,489	2,729	22 (7)
Total Codeine	3,141	2,262	28 (9)
Total Dihydrocodeine	330	223	32 (18)
Pholcodine	40	17	70 (0)
6-AM	1,451	1,358	6 (1)
Ketamine*	63	45	29 (0)
Tramadol*	475	265	44 (5)

In samples where benzoylecgonine was detected, the measured concentration was below the cutoff (150 µg/L) in approximately one fifth of samples. Following cocaine use, the urinary elimination of benzoylecgonine is initially rapid, but is followed by a slower phase meaning benzoylecgonine may be detected up to 3 weeks after use (Nickley *et al.*, 2017). This may be a result of drug leaching out of body tissue. Reporting according to the cutoff will be indicative of recent cocaine use, which may be more clinically relevant for monitoring cocaine use in chronic drug users.

A small proportion of samples had methadone and EDDP detected below the cutoff concentration. Reporting these analytes based on the LoD would be unlikely to offer much clinical benefit in terms of patient management, and may make missed methadone doses harder to identify. When a daily methadone dose is missed, the methadone concentration is likely to be lower in a urine sample collected the following day whilst the EDDP concentration is less affected (Preston *et al.*, 2003). Identifying a missed dose is difficult from analytical results alone due to the variability in methadone concentration. Inter-individual differences mainly arise from either genetic variation in cytochrome P450 enzymes, or drug-drug interactions which cause induction or inhibition of methadone metabolism (Eap *et al.*, 2002).

As 6-AM is a unique marker of heroin/diamorphine use it has significant clinical value. Whilst only a small proportion of samples had 6-AM detected below the cutoff concentration, reporting that 6-AM was detected could influence the treatment and ongoing clinical management of an individual. Conversely, basing the reporting of morphine and codeine on the LoD would be unlikely to influence the treatment of patients in drug rehabilitation.

In samples where dihydrocodeine was detected, the total dihydrocodeine concentration was below the cutoff (300 µg/L) in approximately one third of the cases. The concentration of dihydrocodeine glucuronide was estimated (based on dihydrocodeine calibration) as no reference standard was available at the time of assay development. This may be underestimating the actual concentration in urine, and could account for samples screening negative.

Pholcodine was detected below the cutoff (300 µg/L) in the majority of samples where it was found. Pholcodine may be detected in urine samples 4-6 weeks after ingestion of a single therapeutic dose (Johansen *et al.*, 1990). The observed low concentrations may be a result of the urine sample being collected a long time after drug intake.

A high proportion of samples had tramadol (and metabolites) detected below the cutoff concentration. Inclusion of tramadol in the urine drug screen is intended to identify misuse as opposed to therapeutic use. It is likely that urine tramadol concentration will be higher when it is misused as opposed to when it is used therapeutically. Basing reporting on the LoD may detect more cases of therapeutic use but have minimal impact on the number of abuse cases that are identified.

6.8.3 Conclusions

Results from this audit suggest that basing result interpretation on the LoD as opposed to the cutoff concentrations would affect a large number of results, particularly for amfetamines. Whether it would be clinically useful to report results based on the LoD would largely depend on the clinical circumstances. Reporting results based on cutoff concentrations will largely monitor recent drug use, whereas reporting according to the detection limits will extend the window of detection and may relate to less recent drug use. As the majority of samples received by our laboratory are from drug and rehabilitation services where chronic drug use is being assessed, it is unlikely that significant clinical benefit would be gained from more sensitive, extended detection of drugs. In addition, defining the timeframe of drug misuse may be difficult as exposure to the drug could extend to some weeks ago. Identification of more recent drug misuse may be ascertained by comparison of the creatinine-corrected drug concentration in subsequent samples. Conversely, in cases where single or low-dose administration of a drug is suspected, a high sensitivity screen with analytes reported as ‘detected’ or ‘not detected’ based on analytical detection limits may be beneficial. If analytical detection limits were used, there would be a greater risk of false positives as a result of analyte carryover from analysis of previous samples. Offering a separate ‘high-sensitivity’ assay may be the best approach. In this scenario, a blank sample could be analysed prior to each patient sample to ensure no analyte carryover.

6.8.3.1 Further Work

A reference standard is now available for dihydrocodeine glucuronide, which will enable the accuracy of estimated concentrations to be ascertained. Ideally, dihydrocodeine glucuronide should be incorporated into the assay calibrators to ensure accurate measurement of this analyte.

7 Quantitative Drug Assay: Measurement of naloxone, naltrexone and selected metabolites in plasma, whole blood and urine

7.1 Introduction

7.1.1 Naloxone

Naloxone may be administered as an antidote in cases of acute opioid poisoning. Historically, naloxone has been given by healthcare professionals in clinical settings. Distribution of naloxone to the wider community has been termed 'take-home naloxone'. The concept of take-home naloxone is not new (Strang and Farrell, 1992; Strang *et al.*, 1996), with pilot studies started in the USA in 1996 and in Europe in 1998 (Dettmer *et al.*, 2001). However, it is only recently that the concept has been more widely accepted and more countries are employing schemes for wider naloxone provision to the community. This movement is now reflected by WHO guidelines that recommend countries expand naloxone access to those likely to witness opioid overdoses (World Health Organisation, 2014b).

An increased risk of death as a result of opioid overdose has been identified in certain situations. Particular groups at high risk are; individuals leaving hospital against medical advice after opioid overdose who risk re-entering overdose after naloxone has worn off, and individuals re-entering the community (e.g. after prison release, or discharge from an in-patient treatment programme). At these times an individual's tolerance is decreased and overdose is likely a result of erroneous judgement of dose when returning to opioid use. Provision of naloxone to these high-risk groups may aid reduction of opioid overdose deaths.

Many surveys have shown that a large proportion, typically over 75 %, of injecting heroin users have witnessed another individual experience overdosage (Strang *et al.*, 2000; Seal *et al.*, 2003; Tracy *et al.*, 2005; Gaston *et al.*, 2009; Enteen *et al.*, 2010). This provides a workforce with the potential to intervene and who have demonstrated a willingness to aid in an overdose situation (Wright *et al.*, 2006). Currently, peer members may try to help an individual who has overdosed, but give ineffective or incorrect treatment, e.g. administering cocaine, immersion in a cold bath, inflicting pain (Beswick *et al.*, 2002; Strang *et al.*, 2013). Adequate intervention is only possible if witnesses are aware of the signs of overdose. It has been shown that when adequate training is provided, this group are capable of providing life-saving intervention (Clark *et al.*, 2014; Seal *et al.*, 2005; Strang *et al.*, 2008; Williams *et al.*, 2014)

The effectiveness of take-home naloxone programmes has been demonstrated. Participation in opioid overdose prevention programs is associated with successful

administration to naloxone to reverse opioid overdose (Clark *et al.*, 2014), and the 'risk of opioid-related overdose fatalities is significantly lower in communities providing naloxone distribution and overdose management education than in communities without programme implementation' (EMCDDA, 2015). These findings have been reinforced by a recent systematic review that demonstrated improved survival rates among participants, contributing to reduced overdose mortality rates in the community, and also demonstrated a low rate of adverse events associated with naloxone use (McDonald and Strang, 2016).

7.1.2 Administration of Naloxone

Naloxone (as naloxone hydrochloride in solution, all concentrations as free base) is available in the UK as ampoules at 20 µg/mL (2 mL volume) or 400 µg/mL (1 mL), or in a pre-filled syringe at 1 mg/mL (2 mL) (Joint Formulary Committee, 2015). In cases of opioid overdose, naloxone is ideally administered by intravenous (IV) injection. IV administration provides the fastest clinical response, but a suitable vein may be difficult to identify and this may hinder timely drug delivery. In these cases subcutaneous (SC) or intramuscular (IM) injection may be used, as despite having a slower onset of action a comparable clinical response time may be achieved (Clarke and Dargan, 2002; Wanger *et al.*, 1998).

For the purpose of take-home naloxone, injectable naloxone is not an ideal administration route. The use of needles increases the risk of blood-borne disease transmission and there is a level of skill required to administer the drug correctly. To try and aid users in injecting naloxone, a handheld auto-injector device ('Evzio', Kaleo Inc., Richmond, VA, USA) was approved by the FDA (April 2014). The device talks the user through the procedure to administer a single 0.4 mg dose of naloxone, injected via a retractable needle IM or SC. Greater success of laypersons administering naloxone using the auto-injector has been demonstrated (Edwards *et al.*, 2015). However, the cost of this device is likely to limit its use.

Alternative administration routes are now being explored with the hope to expand the use of naloxone in the community. Three main routes have been identified: intranasal, buccal, and sublingual (Strang *et al.*, 2016). All these routes have the advantage of bypassing the digestive system, and hence first pass metabolism, which will improve naloxone bioavailability. There are few data relating to naloxone pharmacokinetics (PK) via these alternative routes to aid comparison with the injectable routes.

7.1.2.1 Intranasal Naloxone

To date, the use of intranasal naloxone has been explored in greatest detail. The nasal mucosa has attracted attention as an alternative non-invasive drug administration route as it has a large absorptive surface with a rich vascular plexus providing a direct route for drug absorption. This minimises first-pass metabolism and enables the rate of absorption and the plasma concentration achieved to be comparable to IV administration for many drugs (Pires *et al.*, 2009).

Intranasal naloxone has been adopted as an 'off-label' use in some American states, Australia, and some European countries (e.g. Norway) (Doe-Simkins *et al.*, 2009; Hansen, 2014). Typically, 1 mL of 1 mg/mL naloxone hydrochloride solution is sprayed into each nostril, thus 2 mg drug is administered. Studies by Barton *et al.* (2005, 2002) demonstrate clinical efficacy of intranasal naloxone with 83 % individuals having overdosed being shown to respond by regaining consciousness on administration solely by the intranasal route. However, mixed data on naloxone bioavailability via intranasal administration exists with early rat studies suggesting 101 % bioavailability (Hussain *et al.*, 1984), but more recent human studies reporting just 4 % (Dowling *et al.*, 2008). For the Dowling (2008) study, volumes of up to 2 mL were administered in each nostril and significant loss of drug from the nasal cavity either by drainage into the nasopharynx or external run-off may account for the apparent low bioavailability (Wermeling, 2013). A specifically designed nasal product with a more concentrated solution delivered in a smaller volume may give more representative data on the relative bioavailability of intranasal naloxone. A pilot study of the PK of intranasal naloxone commenced in Norway in 2013 (Clinical Trials identifier NCT01939444). The first data from this work suggests bioavailability via the nasal route of 54 % and 52 % for 0.8 mg and 1.6 mg formulations, respectively (Tylleskar *et al.*, 2017).

Two specific nasal spray products have now been manufactured, with one gaining FDA approval on 18 November 2015 ('Narcan', Adapt Pharma Limited, Radnor, Pennsylvania, USA). Data from the approved device demonstrate that the critical PK characteristics of intranasal naloxone are at least equivalent to those when naloxone is administered IM (Table 7.1, Krieter *et al.*, 2016).

Table 7.1 – PK parameters for naloxone after nasal administration, and comparison to IM administration (Krieter *et al.*, 2016)

[a] Mean (SD) for all parameters except Median (Range) for T_{max}

[b] $F\ (\%) = \frac{F^A}{F^B} = \frac{AUC^A}{Dose^A} \times \frac{Dose^B}{AUC^B}$

PK Parameter	IM administration	Intranasal administration		
Dose (mg)	*0.4*	*2*	*4*	*8*
T_{max} (h) [a]	0.4 (0.1-2.1)	0.3 (0.3-1.0)	0.5 (0.2-1.0)	0.3 (0.2-1.0)
C_{max} (µg/L) [a]	0.9 (0.3)	3.1 (1.1)	5.3 (2.4)	10.3 (3.9)
AUC_{inf} (ng*h/mL) [a]	1.8 (0.4)	4.7 (1.4)	8.5 (3.3)	15.8 (3.6)
$t_{1/2}$ (h) [a]	1.3 (0.4)	1.9 (0.7)	2.2 (0.6)	2.2 (0.9)
F (%) [b]	-	52	47	44

Whilst intranasal naloxone may be clinically effective in many cases, damage to the nasal mucosa (e.g. epistaxis, trauma, and deformity) may hinder drug administration. Damage to the mucosa may also be a result of concurrent or past nasal insufflation of other illicit drugs, e.g. cocaine (Ashton and Hassan, 2006). Mechanical problems may also be encountered when trying to use the nasal spray on an individual who is in a seated or supine position (Dowling *et al.*, 2008).

7.1.2.2 Buccal Naloxone

A rodent study reported high bioavailability of naloxone (70 %) from buccal administration (Hussain *et al.*, 1987) thus providing some evidence that buccal administration in humans may be a viable drug delivery route.

Formulation of naloxone in conjunction with buprenorphine as a mucoadhesive buccal film (BUNAVAIL, BioDelivery Sciences) is FDA approved for maintenance treatment of opioid dependence. Manufacturer data does not state the bioavailability of naloxone via buccal administration.

7.1.2.3 Sublingual Naloxone

Sublingual administration of naloxone (dose of 2 mg or greater) has been shown to precipitate withdrawal in opioid-dependent individuals, suggesting sufficient absorption through this route (Preston *et al.*, 1990). The only published PK data for the sublingual route relates to naloxone co-formulated with buprenorphine (Table 7.2, Chiang and Hawks, 2003).

Table 7.2 – PK parameters for naloxone after sublingual administration (in combination with buprenorphine, Chiang and Hawks, 2003)

[a] Mean (SD) for all parameters

PK Parameter	Sublingual Administration	
Dose (mg)	*4*	*8*
T_{max} (h) [a]	0.9 (0.3)	1.0 (0.3)
C_{max} (µg/L) [a]	0.66 (0.53)	0.93 (0.71)
F (%)	9	7

7.1.3 Suboxone

Suboxone is a co-formulation of buprenorphine and naloxone (4:1 by weight, respectively) used for the treatment of opioid addiction. Naloxone is added to deter buprenorphine misuse (e.g. by injection or insufflation, i.e. 'snorted') as symptoms of opioid withdrawal will be induced due to the greater naloxone bioavailability via these routes as opposed to when it is taken sublingually.

Over recent years OST has become more widely available within prisons, with 13 % of the prison population in England and Wales receiving either methadone or buprenorphine (EMCDDA, 2013). The misuse and diversion of OST is known to be a problem, particularly of buprenorphine, which has been named as the third most misused drug in UK prisons (Tompkins *et al.*, 2009). The higher prevalence of buprenorphine diversion may be due to its formulation as a tablet or film, which makes supervising its consumption more difficult compared to that of an oral liquid, e.g. methadone linctus. To minimise diversion and misuse of buprenorphine within prisons, Suboxone may be prescribed which is known to be less desirable than Subutex (buprenorphine only formulation) (Wright *et al.*, 2014). Urinary detection of naloxone and its metabolites may enable differentiation of Suboxone and Subutex use. This may have clinical value in identifying buprenorphine misuse. Urinary detection of naloxone, in addition to buprenorphine, may also enable cases of urine adulteration by direct addition of Suboxone to be identified (Belsey *et al.*, 2014; McMillin *et al.*, 2012).

7.1.4 Naltrexone

Naltrexone has demonstrated success in preventing re-use of heroin after detoxification (Rawson *et al.*, 1979; Shufman *et al.*, 1994). Naltrexone was originally approved as an oral tablet, however its effectiveness is often limited due to poor adherence, with studies consistently showing only approximately a third of patients complete treatment (Minozzi *et al.*, 2011). Adherence to naltrexone therapy is lower than with agonist therapies, probably as a result of naltrexone's lack of reinforcing properties and of withdrawal manifestations on cessation, thus naltrexone therapy has to date been largely confined to highly motivated individuals (Kjome and Moeller, 2011). A sustained release preparation, administered either via depot injection, or implantation, may improve adherence (Comer *et al.*, 2006).

7.1.4.1 Injectable Formulations

To date, three sustained release naltrexone injections have been developed and tested in humans; Depotrex® (Biotek Inc, Woburn, MA), Naltrel® (DrugAbuse Sciences SAS, Paris, France), and Vivitrol® (Alkermes, Waltham, MA) (Bartus *et al.*, 2003). Of these, only Vivitrol has received FDA approval for treatment of opioid addiction (October 2010). Vivitrol is administered I.M. in the gluteal region, and gives sustained plasma naltrexone concentrations for 30 days (Kjome and Moeller, 2011).

7.1.4.2 Implant Formulations

Several different forms of naltrexone implant have been developed by Australian (O'Neil Implant®, Go Medical Industries), Russian (Prodetoxone®, Fidelity Capital), Chinese (Civil Life Scientific Company) and American (Wedgewood Implant®, Wedgewood Pharmacy) companies. Currently none of the implant formulations are approved for clinical use. The implants have comparable or longer duration of action to Vivitrol injection. Typically the duration of action for the developed implants is 6-8 weeks, however the O'Neil Implant is said to last for 5-6 months (Brewer and Streel, 2010; Hulse *et al.*, 2004).

7.1.5 Purpose of Measuring Naloxone and Naltrexone

Most methods for naloxone and naltrexone have been used for PK studies. Clinical measurement of naloxone is not usually necessary as efficacy is based on clinical response (i.e. increased respiratory rate after opioid overdosage) as opposed to reaching a target plasma concentration. Clinical measurement of naltrexone has been employed for TDM, largely for individuals trying to prevent relapse of alcohol dependence

(Danışmant *et al.*, 2012). Naltrexone TDM for individuals being treated for opioid addiction has been advocated (Brünen *et al.*, 2011). As previously mentioned, detection of naloxone and its metabolites in urine may be beneficial for identifying the misuse of Suboxone (Section 7.1.3).

7.1.6 Analytical Methods for the Measurement of Naloxone and Naltrexone

A wide range of analytical methods have been reported for the measurement of naloxone and naltrexone. These include radioimmunoassay, TLC, HPLC coupled with electrochemical detection (ED), ultra-violet detection (UV) or mass spectrometric detection (MS), and GC coupled with flame ionisation detection (FID), electron capture detection (ECD) or MS detection.

Naloxone and naltrexone were both developed in the 1960s. Analytical methods at this time used radioimmunoassay. The radioimmunoassay used for naloxone measurement lacked selectivity, with studies demonstrating that whilst naloxone glucuronide and nornaloxone were not recognized by the antibody, naltrexone and 6-hydroxynaloxone were able to displace naloxone-^{3}H from the antibody (Berkowitz *et al.*, 1975).

Early LC methods used either UV or ED for the measurement of naloxone (Albeck *et al.*, 1989; Reid *et al.*, 1993). ED offers greater sensitivity when compared to UV detection, however UV is often more robust with ED susceptible to contamination and often requiring longer equilibration time (Brünen *et al.*, 2010). Early GC methods used FID (Cone *et al.*, 1974) or ECD (Verebey *et al.*, 1980) for measurement of naltrexone.
More recently, the coupling of chromatographic techniques to mass spectrometry has led to huge advances in both assay selectivity and sensitivity. GC-MS methods are widely used in drug analysis. However they often require an extensive sample preparation step, which may take days to complete. Many analytes require derivatisation to enable their detection by GC-MS. Problems can arise in converting an analyte to a single suitable derivative, which may limit assay sensitivity. The derivatization of naltrexone for GC-MS is associated with partial derivatization of the enolic form of the ketone group (Nelson *et al.*, 1993), for example. LC-MS offers a more versatile methodology, with no derivatisation required. LC-MS (and LC-MS/MS) methods have been widely adopted for measuring naloxone and naltrexone (Brünen *et al.*, 2010; Clavijo *et al.*, 2008; Eckart *et al.*, 2015; Jiang *et al.*, 2011; Moreno-Vicente *et al.*, 2015; Slawson *et al.*, 2007; Yun *et al.*, 2007). Most methods employing mass spectrometry as a detection method are targeted (e.g. SIM, SRM) which gives high selectivity. The

disadvantage of this approach is the inability to detect co-medication and look retrospectively for other analytes, e.g. minor metabolites. Collection of full scan data captures the maximum amount of information from a sample analysis, but in the past this has meant a compromise in assay selectivity. More recently, the advent of high-resolution mass spectrometers coupled with accurate mass measurement has led to improved selectivity when capturing full scan data.

7.1.6.1 Limits of Analytical Detection and Quantitation

As analytical technology has advanced, analytes can be detected and measured at lower concentrations. Using LC-ED detection limits reached 1 µg/L for naloxone (Reid *et al.*, 1993) and 0.25 µg/L for naltrexone (Davidson *et al.*, 1996) in plasma. The current lowest published quantitation limits for each analyte in plasma are listed in Table 7.3, and all utilise LC-MS/MS methods.

Table 7.3 – Lowest quantitation limits published for naloxone, naltrexone and metabolites in plasma using LC-MS/MS

Analyte	LLoQ (µg/L)	Reference
Naloxone	0.001	Liu *et al.* (2016)
Nornaloxone	0.5	Fang *et al.* (2009)
6-β-Naloxol	0.4	Jiang *et al.* (2011)
Naloxone glucuronide	0.5	Dong *et al.* (2013); Jiang *et al.* (2011)
Naltrexone	0.005	Clavijo *et al.* (2008)
6-β-Naltrexol	0.005	Clavijo *et al.* (2008)

7.1.6.2 Published Analyte Stability

A summary of published stability data for naloxone, naltrexone and their metabolites in human plasma is given in Table 7.4. To date no stability data have been published for 6-β-naloxol in human plasma. The stability of 6-β-naloxol in mouse plasma has been investigated, with results showing stability for 26 h at room temperature and 40 days at -20 ^{o}C (Jiang *et al.*, 2011).

Table 7.4 – Naloxone/naltrexone assay: Analyte stability in human plasma
[a] stored at -80 °C, [b] stored at -70 °C

Analyte	Study	Storage condition [duration of analyte stability]		No. of freeze-thaw cycles analyte documented as stable
		Ambient [h]	-20 °C [days]	
Naloxone	Liu *et al.* (2016)	6.2	606	4
	Fang *et al.* (2009)	24	468	3
	Moody *et al.* (2002)	24	-	3
	Moreno-Vicente *et al.* (2015)	-	93 [a]	3
Nornaloxone	Fang *et al.* (2009)	24	468	3
Naloxone glucuronide	Dong *et al.* (2013)	6	90[b]	-
Naltrexone	Clavijo *et al.* (2008)	12	-	3
	Chan *et al.* (2001)	-	21 42 [a]	-
	Huang *et al.* (1997)	24	-	2
	Slawson *et al.* (2007)	24	-	3
	Yun *et al.* (2007)	6	30[b]	3
6-β-Naltrexol	Clavijo *et al.* (2008)	12	-	3
	Chan *et al.* (2001)	-	21 42[a]	-
	Slawson *et al.* (2007)	24	-	3
	Yun *et al.* (2007)	6	30[b]	3

7.1.7 Aims

The aim of method development was to simultaneously assay naloxone, naltrexone and selected metabolites (nornaloxone, 6-β-naloxol, naloxone-3-glucuronide, and 6-β-naltrexol). The method will be validated for plasma and whole blood for use in PK studies, and where possible the LoD for analytes will be improved compared to published methods. The method will also be validated for urine, and will be utilised to attempt to differentiate Suboxone and Subutex use.

7.2 Materials and Methods

7.2.1 Chemicals and Reagents

Naloxone-D_5, naloxone-3-glucuronide, naloxone-3-glucuronide-D_5, 6-β-naloxol, 6-β-naltrexol, naltrexone-D_4, and naltrexone-D_7 were supplied as powders (Alsachim, Illkirch-Graffenstaden, France). Naloxone, naloxone *N*-oxide, and 6-AM were supplied as 1 g/L solutions and naltrexone hydrochloride as powder (Sigma Aldrich, Poole, UK). HPLC grade acetone and 2-propanol were all from Rathburn (Walkerburn, UK). Ammonium acetate, ammonium bicarbonate, and trichloroacetic acid were from Sigma-Aldrich, and coarsely filtered, pooled human serum was from Sera Labs (Haywards Heath, UK). 1.5 mL Eppendorf tubes were from Elkay (Basingstoke, UK). For all other chemicals and reagents see Section 2.2.1.

7.2.2 Instrumentation

All instrumentation is detailed in Section 2.2.2.

7.2.3 Liquid Chromatography

System eluents were as follows: (A) 0.1 % (v/v) formic acid in water, and (B) 0.1 % (v/v) formic acid in acetonitrile:methanol (1+1). Prepared samples were directly injected (100 µL) onto a Raptor Biphenyl HPLC column (2.7 µm aps, 100 x 2.1 mm I.D., Restek, High Wycombe, UK). The column was fitted with an UltraShield 0.2 µm pre-column filter (Restek), and maintained at 30 °C. Gradient elution (total flow 0.6 mL/min) was used. The starting conditions were 98 % A 2 % B ramped to 75 % A 25 % B over 2 min, and then ramped to 10 % A 90 % B over 1.5 min, held for 1.5 min, then returned to initial conditions for 2 min to re-equilibrate. The total analysis time was 7 min.

7.2.4 Mass Spectrometry

MS was carried out in positive ionisation mode using heated ESI [spray voltage 4.5 kV; temperatures: vaporiser 450 ^{o}C, capillary 250 ^{o}C; auxiliary, sheath and sweep gases 15, 55, and 0 (arbitrary units, respectively), S-lens voltage 90 V]. Full-scan MS data were acquired using a resolution setting of 35,000 defined as FWHM at *m/z* 200, with a scan range of 200-700 *m/z* (Orbitrap settings: maximum injection time 10 ms, AGC 1 x 10^5 ions).

7.2.5 Preparation of Calibration and Internal Quality Control Solutions

Individual stock solutions of each analyte (all 100 mg/L except; 91.6 mg/L naloxone-3-glucuronide; 93 mg/L naloxone-3-glucuronide-D_5; 75.3 mg/L naltrexone-D_4; 102.3 mg/L naltrexone-D_7) were prepared in methanol. All stock solutions were stored at -20 °C. Nominal concentrations of analytes present in plasma and urine calibrator and IQC solutions are given in Table 7.5.

Table 7.5 – Naloxone/naltrexone assay: Analyte concentration in a) plasma, and b) urine, calibrator and IQC solutions

	Nominal concentration (µg/L)									
	Calibrator							IQC		
	1	2	3	4	5	6	7	A	B	C
a) Plasma										
Naloxone 6-β-Naloxol	0.05	0.1	0.2	0.5	1	5	10	0.15	2.5	7.5
Nornaloxone Naltrexone 6-β-Naltrexol	0.1	0.2	0.4	1	2	10	20	0.3	5	15
Naloxone-3-glucuronide	0.5	1	2	5	10	50	100	1.5	25	75
b) Urine										
Naloxone 6-β-Naloxol Nornaloxone Naltrexone 6-β-Naltrexol Naloxone-3-glucuronide	10	50	100	250	500	750	1000	20	300	550

7.2.5.1 Plasma

Stock solutions were diluted as appropriate with methanol to give working solutions A (50 µg/L each naloxone and 6-β-naloxol; 100 µg/L each nornaloxone, naltrexone, and 6-β-naltrexol; 500 µg/L naloxone-3-glucuronide) and B (500 µg/L each naloxone and 6-β-naloxol; 1000 µg/L each nornaloxone, naltrexone, and 6-β-naltrexol; 5000 µg/L naloxone-3-glucuronide). Appropriate volumes of working solutions were evaporated to dryness in 10 mL volumetric flasks under a gentle stream of compressed air and reconstituted with pooled human plasma to prepare calibration standards and IQC solutions. After thorough mixing and equilibration (24 h, 2-8 °C), the prepared calibration and IQC solutions were stored in approximately 600 µL portions in 2 mL polypropylene screw-top tubes at -20 °C until required.

7.2.5.2 Urine

Appropriate volumes of stock solutions were evaporated to dryness in 10 mL volumetric flasks under a gentle stream of compressed air and reconstituted with analyte-free human urine to prepare calibration standards and IQC solutions. After thorough mixing and equilibration (24 h, 2-8 °C), the prepared calibration and IQC solutions were stored in approximately 250 µL portions in 2 mL polypropylene screw-top tubes at -20 °C until required.

7.2.6 Internal Standard Solutions

The plasma working IS solution containing 0.2 µg/L of both naloxone-D_5 and naltrexone-D_7, and 2 µg/L of naloxone-3-glucuronide-D_5, was prepared by appropriate dilution of the stock solutions with acetonitrile containing 0.1 % (v/v) formic acid. The solution was stored and used at -20 ^{o}C.

The urine working IS solution containing 10 µg/L of naloxone-D_5, naltrexone-D_7 and naloxone-3-glucuronide-D_5 was prepared by appropriate dilution of the stock solutions with eluent A. The solution was stored at 2-8 °C.

7.2.7 Sample Preparation

For blood samples, samples/calibrators/IQCs (250 µL) were mixed with 750 µL working internal standard into Eppendorf tubes. The tubes were capped, vortex-mixed (1 min) and centrifuged (13,500 rpm, 5 min). The upper layer was transferred to a new Eppendorf tube and evaporated to dryness under a stream of compressed air at 40 ^{o}C. The sample was reconstituted in 125 µL eluent A (vortex-mix 30 s), and transferred to a 0.2 mL glass vial. The vials were capped and transferred to a pre-cooled (10 ^{o}C) autosampler tray.

For urine samples, centrifuged samples/calibrators/IQCs (50 µL) were diluted with 450 µL working IS solution directly into HPLC vials. The vials were capped and transferred to a pre-cooled (10 ^{o}C) autosampler tray.

7.2.8 Assay Calibration and Acceptance Criteria

Calibration standards and matrix blanks were included at the beginning and end of each batch analysis, with IQCs included (i) after the first set of calibration standards and immediately before the last set, and (ii) after every ten injections throughout the sequence. Samples with concentrations exceeding the calibration range for an analyte were diluted as appropriate with analyte-free matrix and re-assayed. Assay acceptance

criteria were (i) linear (R^2 >0.98) calibration curves for each analyte, and (ii) IQC values within ±15 % nominal concentrations for all analytes.

7.2.9 Method Validation Protocol

The method was validated according to the FDA/CDER guidance for bioanalytical method validation (FDA/CDER, 2013). Intra- and inter-assay accuracy (% nominal concentration) and precision (% RSD) were assessed for each analyte at three concentrations through replicate analysis of the IQC solutions (N = 5) on the same day and singleton analysis on 5 different days, respectively. The LLoQ was ascertained through replicate analysis of the lowest calibrator and was based upon a signal-to-noise ratio >10, and RSD <20 % (N = 5). To assess the linearity for each analyte, a high concentration sample (all analytes 500 µg/L in plasma, 10,000 µg/L in urine) was diluted using blank matrix to within the calibration range.

To assess analyte recovery in plasma, aqueous solutions were prepared with all analytes and internal standards at 50 µg/L and at 100 µg/L. These samples were prepared and analysed as described except that 0.1 % formic acid in acetonitrile was added in place of IS solution. The mean peak areas (N = 3) obtained from analyses of the extracts of the aqueous solution (50 µg/L all analytes and internal standards) were compared to the mean peak areas (N = 3) obtained from direct injection of an aqueous solution (100 µg/L all analytes and internal standards, to be comparable with extracted samples that undergo two-fold concentration). Analyte recovery was not necessary for urine samples as sample preparation only involved dilution with IS solution.

Initially, matrix effects were assessed qualitatively in plasma using the post-column infusion method (Bonfiglio *et al.*, 1999). Analyte-free plasma from 10 independent sources was analysed. These samples were prepared and analysed as described except that 0.1 % formic acid in acetonitrile was added in place of IS solution, and the detector response for each analyte was monitored whilst a methanolic solution containing all analytes and internal standards (1 mg/L, 20 µL/min) was infused by syringe post-column. To further quantify matrix effects and ascertain if the internal standards selected were appropriate, addition experiments were undertaken in plasma, whole blood, and urine. Solutions containing all analytes and internal standards (50 µg/L) were prepared in (i) analyte-free human plasma/whole blood/urine from 10 independent sources, and (ii) deionised water. Samples were prepared and analysed as described

above except that 0.1 % formic acid in acetonitrile for the plasma/blood method and 0.1 % formic acid in water for the urine method were added in place of the IS solutions.

Analyte carryover was assessed through consecutive analysis of a sample containing low (L) and high (H) analyte concentrations in the order L, L, L, H, H, H, L, L, L (L: all analytes 0.5 µg/L and H: all analytes 50 µg/L in plasma; L: all analytes 10 µg/L and H: all analytes 2000 µg/L in urine).

7.2.9.1 Stability Studies

Stability studies were conducted in plasma only. The stability of all analytes was assessed through analysis of portions of IQC solutions stored (i) at ambient temperature for 48 hours, (ii) refrigerated for 2 weeks, and (iii) after multiple (N = 4) freeze-thaw cycles. Temperatures were monitored using automated software with measurements every 15 min at each condition (Comark, Norwich UK). For each storage condition, analyte concentrations were assessed against calibrators stored frozen and thawed for the first time immediately prior to use. Three separate portions of each IQC were analysed under each condition, and the mean concentration used to assess stability. Analyte instability was defined as a deviation >20 % from the expected concentration.

7.2.10 Differentiating Suboxone and Subutex Use

Fourteen urine samples from patients known to be taking Suboxone were analysed to provide information on the typical urinary naloxone and metabolite concentrations achieved during routine Suboxone therapy. Buprenorphine and metabolites were measured in these samples using the urine drug screening method (Chapter 2).

7.2.11 Patient Samples and Ethics Considerations

Excess blood from samples submitted for TDM and excess urine from samples submitted for urine drug screening were used to ascertain matrix effects and to ascertain if the method was fit-for-purpose; these samples would normally have been discarded following the normal ethics guidelines. No records kept during method development permitted identification of patients. For differentiating Suboxone from Subutex use, excess urine submitted for drug screening from individuals known to be taking Suboxone were sent anonymised to our laboratory from St. James's University Hospital (Leeds, UK), and urine samples sent to King's College Hospital for routine drug screening which stated Subutex as a prescribed medicine on the request form were used to assess any potential interference with the naloxone assay.

7.3 Results and Discussion

7.3.1 Liquid Chromatography Method Development

7.3.1.1 Choice of LC column

Most published methods for measurement of naloxone and naltrexone have used reverse-phase chromatography, typically with a C18-modified silica column (Table 7.6). For the measurement of naloxone glucuronide, hydrophilic interaction liquid chromatography (HILIC) has also been reported (Dong *et al.*, 2013).

Initially an Accucore Phenyl Hexyl column and a Hypersil Gold C18 column (both 100 x 2.1mm, ThermoFisher Scientific) were evaluated, but naloxone-3-glucuronide did not retain well on either column. An alternative column, Hypercarb (porous graphitised carbon, ThermoFisher Scientific), was investigated due to its capability to retain more polar analytes. Good retention of all analytes on a Hypercarb column was achieved and an LC method optimised, but when switching to a newly manufactured column the peaks and column performance were not reproducible. Due to this lack of robustness, a third stationary phase with a biphenyl ligand (Raptor Biphenyl 2.7 µm, 100 x 2.1mm, Restek) was evaluated. Development of the LC method using the biphenyl column is detailed below.

Table 7.6 – Some published LC methods for the detection of naloxone and naltrexone

Abbreviations: aps – average particle size, I.D. – Internal diameter, C18 – octadecyl, CN – cyano, RP – reverse phase.

Analytes in method	LC Column Details	Reference
Naltrexone, 6-β-naltrexol	C18 (50 x 4.6 I.D. mm; 1.8 µm aps, Agilent XDB)	Dodou *et al.* (2015)
Naltrexone, 6-β-naltrexol	C18 (125 x 4 I.D. mm; 5 µm aps, ThermoFisher Hypersil)	Heinälä *et al.* (2012)
Naltrexone, 6-β-naltrexol	C18 (150 x 2.1 I.D. mm; 5 µm aps, ThermoFisher Hypersil)	Brünen *et al.* (2010)
Naltrexone, 6-β-naltrexol	C18 (150 x 4.6 I.D. mm; 5 µm aps, Supelco Supelcosil)	Zuccaro *et al.* (1991)
Naltrexone, 6-β-naltrexol	CN (30 x 4.6 I.D. mm; 5 µm aps, Phenomenex Luna)	Clavijo *et al.* (2008)
Naltrexone, 6-β-naltrexol	Phenyl (100 x 4.6 I.D. mm; 3 µm aps, YMC)	Davidson *et al.* (1996)
Naloxone, naltrexone & 6-β-naloxol	Phenyl hexyl (100 x 2.1 I.D. mm;1.8 µm aps, Zorbax Eclipse)	Eckart *et al.* (2015)
Naloxone & naltrexone	Phenyl (150 x 4.6 I.D. mm; 5 µm aps, Waters X-Bridge)	Moreno-Vicente *et al.* (2015)
Naloxone	C18 (50 x 2.1 I.D. mm; 2.5 µm aps, Waters X-Bridge)	Krieter *et al.* (2016)
Naloxone	C18 (50 x 2.1 I.D. mm; 3 µm aps, Imtakt Unison)	Liu *et al.* (2016)
Naloxone	C18 (100 x 2.1 I.D. mm; 4 µm aps, Phenomenex Gemini)	Heikman *et al.* (2014)
Naloxone	C18 (50 x 2 I.D. mm; 3 µm aps, YMC)	Moody *et al.* (2002)
Naloxone	C18 (150 x 4.6 I.D. mm; 5 µm aps, Alltech Econosphere)	Reid *et al.* (1993)
Naloxone, 6-β-naloxol, naloxone-3-glucuronide	C18 (50 x 2.1 I.D. mm; 5 µm aps, ThermoFisher Aquasil)	Jiang *et al.* (2011)
Naloxone & naloxone-3-glucuronide	Polar RP (150 x 2 I.D. mm; 4 µm aps, Phenomenex Synergi)	Al-Asmari and Anderson (2007)
Naloxone, nornaloxone	C18 (50 x 2.1 I.D. mm; 3.5 µm aps, Waters XTerra)	Fang *et al.* (2009)
Naloxone-3-glucuronide	HILIC 100A (50 × 2.1 I.D. mm; 2.6 µm aps, Phenomenex Kinetex)	Dong *et al.* (2013)

7.3.1.2 Aqueous eluent selection

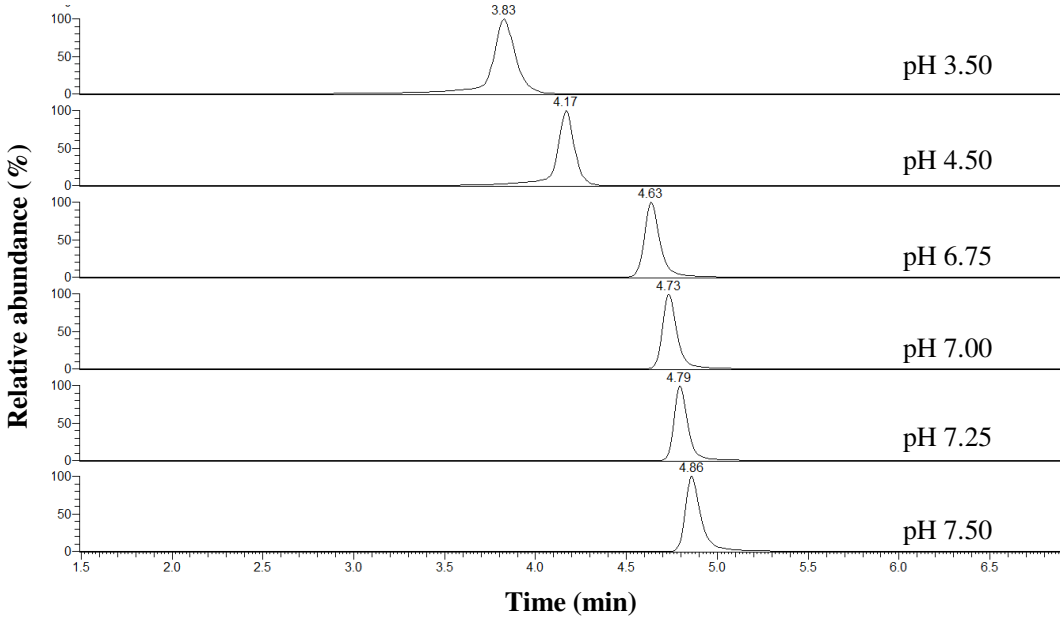
When first testing the biphenyl column, a simple gradient (2-100 % organic eluent [0.1 % (v/v) formic acid in methanol] over 6 minutes) was used. The column temperature was maintained at 50 ^{o}C, and the LC flow rate was 0.4 mL/min. Initially, the aqueous phase tested was 0.1 % (v/v) formic acid in deionised water. Under these conditions nornaloxone, the first eluting peak, was not completely retained on the column. Nornaloxone lacks the branched alkene/cyclopropane moiety that the other analytes possess, which may account for less interaction with the biphenyl column. The biphenyl column is stable over a pH range of 1.5-8.0. To attempt to improve retention of nornaloxone, the addition of volatile ammonium salts (all 10 mmol/L) to the aqueous phase was investigated over the pH range 3.0-7.5 (Table 7.7).

Table 7.7 – Ammonium salts used to investigate the effect of pH on analyte retention

Ammonium salt	Buffering range (pH)	pH range investigated
Formate	2.7-4.7	3.00-4.00
Acetate	3.8-5.8	4.00-5.00
Bicarbonate	6.6-8.6	6.75-7.50

Retention of nornaloxone was improved at higher pH, with greatest retention at pH 7.5 (Figure 7.1). Unfortunately, whilst ammonium bicarbonate is LC-MS compatible it was found that the biphenyl column showed increased back pressure rapidly when using this method. Ammonium bicarbonate is not very soluble in methanol or acetonitrile, and as a result was likely precipitating in the column. Unfortunately, no other LC-MS compatible buffers are capable of buffering at higher pH. As a result the aqueous eluent used was returned to 0.1 % (v/v) formic acid in water, as both ammonium formate and ammonium acetate caused significant peak fronting, and alternative approaches to improve nornaloxone retention were considered.

Figure 7.1 – Extracted ion chromatograms (*m/z* 288.1230) to show the influence of pH on LC retention of nornaloxone [Biphenyl (100 x 2.1 I.D. mm) column, Restek] (see Section 7.3.1.2)



7.3.1.3 LC flow rate and column temperature

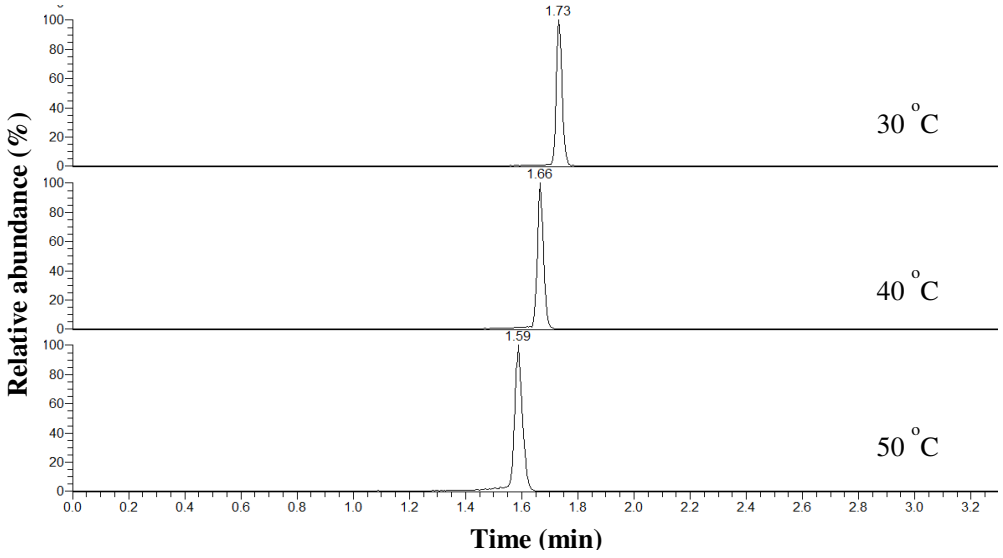
Analyte retention may be influenced through LC flow rate and column temperature. A lower flow rate will increase analyte retention, but may emphasise peak broadening. When using ESI, a lower flow rate also offers the benefit of improved assay sensitivity. Increased column temperature may reduce analyte retention enabling analysis time to be shortened, and may result in sharper chromatographic peaks due to more efficient solute transfer between the mobile phase and the stationary phase (Vanhoenacker *et al.*, 2010). To assess the impact of LC flow rate, the column was held at 30 ^{o}C whilst the total LC flow rate was altered (range 0.3-0.6 mL/min). Improved MS response was noted at lower flow rate (Table 7.8), however peak shape deteriorated significantly and thus the flow was maintained at 0.6 mL/min. The optimum column temperature over 30-50 ^{o}C was then investigated in steps of 5 ^{o}C. Fronting of the nornaloxone peak worsened at temperatures over 40 ^{o}C, and thus the column oven temperature was kept at 30 ^{o}C (Figure 7.2).

Table 7.8 – Naloxone/naltrexone assay: The impact of decreasing LC flow rate on analyte peak areas

The relative analyte peak area (%) to the observed peak area with the LC flow rate at 0.6 mL/min is given.

Analyte	LC flow rate (mL/min)		
	0.5	0.4	0.3
Nornaloxone	136 %	155 %	192 %
Naloxone-3-glucuronide	131 %	158 %	206 %
Naloxone	115 %	130 %	165 %
6-β-Naloxol	119 %	132 %	153 %
Naltrexone	115 %	136 %	164 %
6-β-Naltrexol	117 %	134 %	158 %

Figure 7.2 – Extracted ion chromatograms (*m/z* 288.1230) to show the influence of column temperature on the retention of nornaloxone [Biphenyl (100 x 2.1 I.D. mm) column, Restek] (see Section 7.3.1.3)



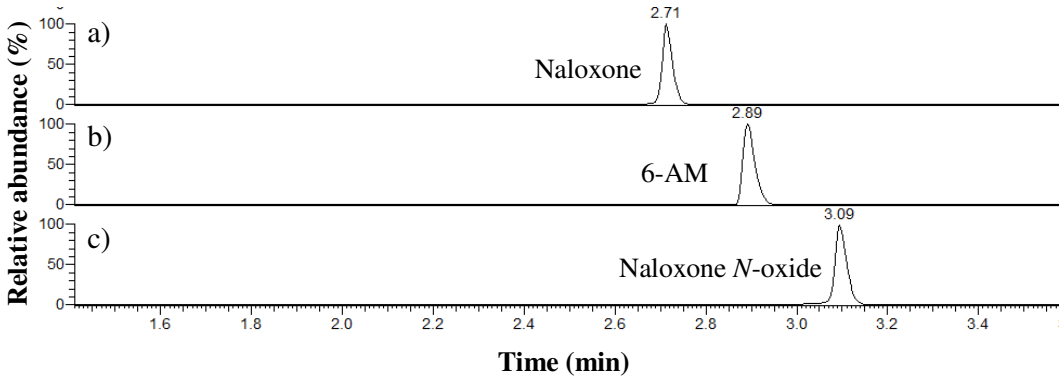
7.3.1.4 Organic eluent selection

Three organic eluents were investigated; 0.1 % (v/v) formic acid in i) acetonitrile, ii) acetonitrile:methanol (1+1), and iii) methanol. The best peak shape was observed when using solely acetonitrile, however chromatographic resolution was improved when using methanol. Acetonitrile:methanol (1+1) offered a good compromise and was thus used for the developed method.

Alteration of the organic eluent was also important for improving assay selectivity. 6-AM is isobaric with naloxone (*m/z* 328.1543). As 6-AM is the primary metabolite of diamorphine, it is possible that 6-AM and naloxone could both be present in a sample. To ensure accurate quantitation of naloxone, it was important that 6-AM was chromatographically resolved from naloxone. This was achieved through alteration of the organic phase, with 0.1 % (v/v) formic acid in acetonitrile:methanol (1+1) providing good resolution (Figure 7.3).

Other possible sources of interference in the assay are *N*-oxides. *N*-Oxides can undergo in-source decomposition to their parent compound and thus interfere with analyte quantitation if not resolved chromatographically, or removed by sample preparation procedures. The extent of *N*-oxide deoxygenation using ESI is significantly lower than when using APCI (Ibrahim *et al.*, 2013). However, deoxygenation of naloxone *N*-oxide may occur ($[(M+H)-O]^+$, *m/z* 328.1544). Naloxone (2.71 min) is chromatographically resolved from the *N*-oxide (3.09 min) in the method developed (Figure 7.3). Naloxone *N*-oxide is an impurity in pharmaceutical formulations of naloxone, and may also be a potential metabolite. There is nothing to suggest metabolism of naltrexone to naltrexone *N*-oxide, and no reference material is available commercially.

Figure 7.3 – Extracted ion chromatograms to show chromatographic resolution of a) naloxone (*m/z* 328.1543) from b) 6-AM (*m/z* 328.1543) and c) naloxone *N*-oxide (*m/z* 344.1493) [Biphenyl (100 x 2.1 I.D. mm) column, Restek]



7.3.2 Mass Spectrometry Method Development

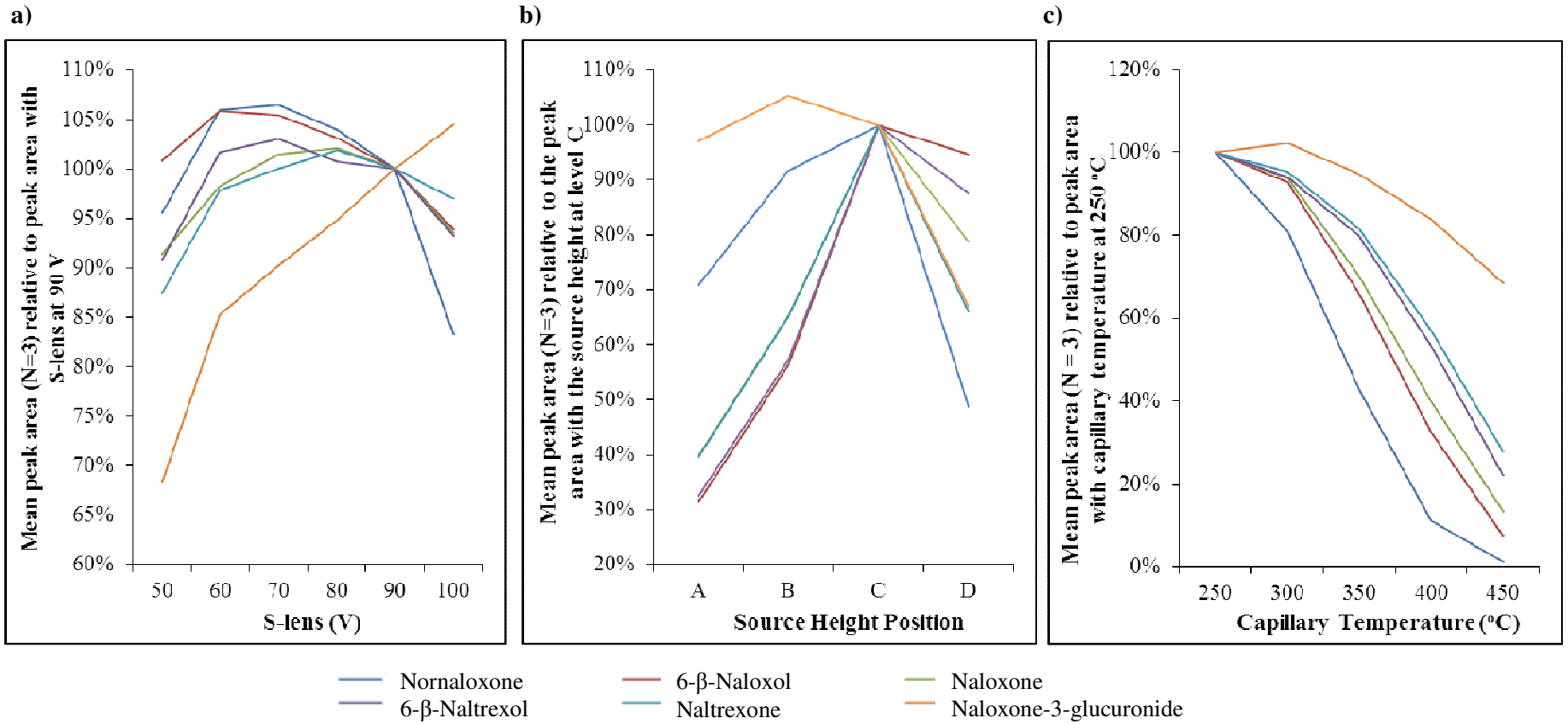
7.3.2.1 Optimisation of Source Conditions

An aqueous solution containing all analytes (100 µg/L) was analysed (N = 3) under different instrument settings, and the mean peak area compared to ascertain the optimum settings. The parameters assessed were: S-lens voltage (50-100 V, in 10 V steps), distance of the ESI probe from the mass spectrometer source (levels A-D, as described in Section 2.3.3.2), and capillary temperature (250-450 ^{o}C, in 50 ^{o}C steps).
Most analytes were little affected by the change in S-lens voltage, with the exception of naloxone-3-glucuronide, which had a markedly increased response at higher S-lens voltages. For most analytes, response peaked at probe height setting C, and analyte response declined as capillary temperature increased (Figure 7.4).

To ascertain the other source conditions, including gas flow rates, spray voltage and vaporiser temperature, the instrument software was used to give the default settings based upon a LC flow rate of 0.6 mL/min.

Figure 7.4 – Optimisation of source conditions for naloxone/naltrexone assay: the influence of a) S-lens voltage, b) ESI probe height, and c) capillary temperature on analyte peak area (see Section 7.3.2.1)

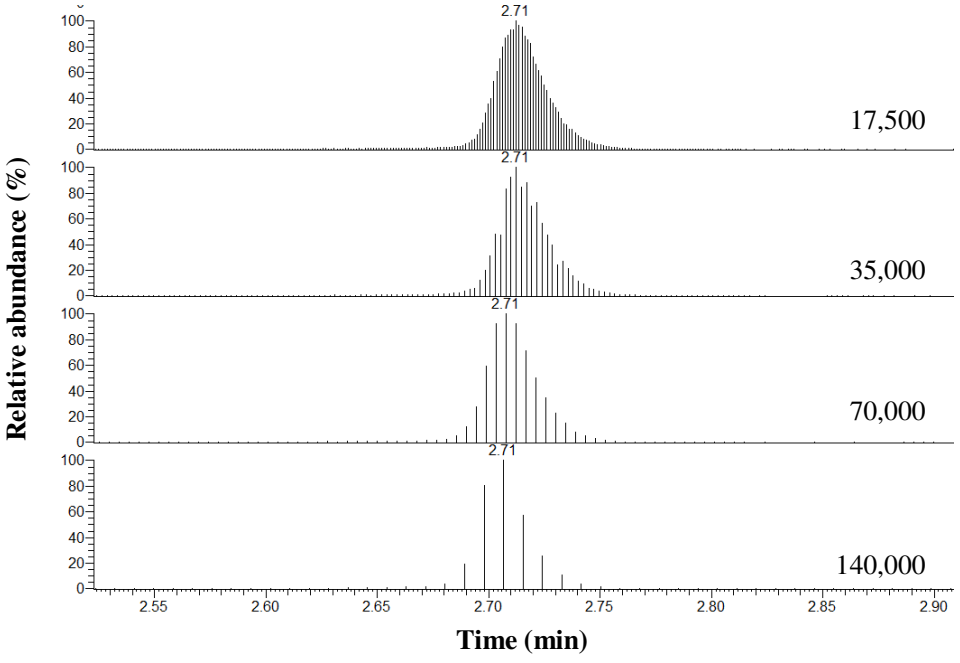
The mean peak areas (N=3) of analytes in an aqueous solution (all 100 µg/L) were compared using different source settings to ascertain the optimum parameters.



7.3.2.2 Mass Resolution

Mass resolution relates to the ability of a mass spectrometer to separate peaks of one mass-to-charge ratio from another, with higher resolution giving improved separation. However, increasing mass resolution increases the time required for each scan. For accurate quantitation it is suggested that a minimum of 10 scans are collected across each chromatographic peak. To assess the impact of increasing mass resolution on the number of MS scans collected over a chromatographic peak, an aqueous solution (all analytes 100 µg/L) was analysed using different resolution settings (Figure 7.5). As the chromatographic peaks were narrow, the resolution selected for the method was 35,000, which provided an adequate number of data points per peak.

<u>Figure 7.5</u> – Extracted ion chromatograms (*m/z* 328.1543) to show the reduction in MS scans collected over the naloxone peak due to an increase in mass resolution (theoretical resolution stated, defined as FWHM at *m/z* 200)



7.3.2.3 MS Scan Experiments

A key parameter for naloxone/naltrexone assay for PK purposes is sensitivity. The highest sensitivity will be achieved from quantifying on the parent ion as opposed to product ions, thus either a full scan or SIM may be used. Full scan data were selected for the method as this gives the potential to interrogate data retrospectively if necessary. This may be beneficial for looking for other minor metabolites, e.g. naloxone *N*-oxide, and co-prescribed medications.

Inclusion of an MS^2 scan, in addition to the full scan, to provide further analyte confirmation through the presence of product ions was investigated. The scan number across the peak, particularly at low analyte concentration, was too low to justify inclusion of this additional experiment. As accurate mass is being used, the certainty of analyte identification is greatly improved as compared to using nominal mass, especially when coupled with other confirmatory parameters such as isotope pattern and retention time.

7.3.3 Sample Preparation

The physicochemical properties of each analyte (Table 7.9) were studied prior to devising a sample preparation procedure. The *p*Ka is important as it gives information on the charge of each analyte species at differing pH values. Log P (partition coefficient) was used to study how the polarities of analytes varied with pH, which is particularly relevant to liquid-liquid extraction.

Table 7.9 – Naloxone/naltrexone assay: Physicochemical properties of the analytes studied (theoretical values calculated using MarvinSketch, version 6.3.0, ChemAxon Ltd)

Analyte	*p*Ka	Log P
Naloxone	7.84	1.62
Nornaloxone	9.36	0.51
6-β-Naloxol	8.54	1.21
Naloxone-3-glucuronide	8.52	-0.33
Naltrexone	8.88	1.67
6-β-Naltrexol	9.47	1.26

In addition to studying the properties of the analytes, a literature search was performed to ascertain the sample preparation methods used previously. Protein precipitation has been used either as a sole preparation method (Brünen *et al.*, 2010; Dong *et al.*, 2013), or in conjunction with a further sample preparation step (Clavijo *et al.*, 2008). Liquid-liquid extraction has been widely used, but not for all analytes; no published method has used this technique for naloxone-3-glucuronide or for nornaloxone (Davidson *et al.*, 1996; Heikman *et al.*, 2014; Heinälä *et al.*, 2012; Liu *et al.*, 2016; Moody *et al.*, 2002; Nelson *et al.*, 1993; Verebey *et al.*, 1980). Solid phase extraction, typically with C18 cartridges, has also been used (Albeck *et al.*, 1989; Chan *et al.*, 2001; Fang *et al.*, 2009; Huang *et al.*, 1997; Reid *et al.*, 1993; Toennes *et al.*, 2004; Ventura *et al.*, 1988; Zuccaro *et al.*, 1991).

7.3.3.1 TurboFlow

TurboFlow coupled to LC-MS has been used for opioid analysis in plasma, but naloxone and naltrexone have not been analysed using this sample preparation technology. Three different TurboFlow columns were evaluated: Cyclone-P, C18-P-XL and Cyclone-MAX (ThermoFisher Scientific). All columns have reverse phase chemistry, with the Cyclone-MAX also having anion exchange capacity. Initial studies showed the C18-P-XL column did not retain naloxone-3-glucuronide and thus it was

not studied further. Improved retention of naloxone-3-glucuronide was observed using the Cyclone-MAX column, as opposed to the Cyclone-P, and thus conditions were optimised using the former column.

Retention of analytes on the Cyclone-MAX TurboFlow column during the loading step was investigated by setting up a simple method that changed sequentially through deionised water, methanol [formic acid in methanol (0.1 % v/v)] and mixed solvent (acetonitrile:2-propanol:acetone; 2+2+1) eluents, reflecting the loading, transfer, and wash stages respectively, prior to re-equilibration for the next analysis. No analytical column was used, and flow was directed constantly to the MS, rather than to waste. Ideally peaks should be observed during the transfer step, and not in the loading or washing stages.

The aqueous eluent was varied to investigate the impact of loading pH on analyte retention, with three pH values being studied: 3 (0.1 % v/v formic acid in deionised water), 6 (10 mmol/L aqueous ammonium acetate), and 9 (10 mmol/L aqueous ammonium carbonate). An aqueous solution containing all analytes (100 µg/L) was analysed (N = 3) and mean peak areas in the loading, transfer and washing steps measured.

Retention of all analytes, particularly naloxone-3-glucuronide, was best when loading at pH 9. Retention of naloxone-3-glucuronide on the Cyclone-MAX column can be attributed mainly to anion exchange, with negatively-charged species predominating at pH 9 (Table 7.10). With all other analytes the predominant retention mechanism is through reverse phase chemistry as only a small proportion of the analyte will carry a negative charge at pH 9.

Table 7.10 – Percentage of analyte species negatively charged at pH 9 (theoretical values calculated using MarvinSketch, version 6.3.0, ChemAxon Ltd)

Analyte	Negatively-charged species (%)
Naloxone	7.4
Nornaloxone	1.9
6-β-Naloxol	5.1
Naloxone-3-glucuronide	93.8
Naltrexone	4.3
6-β-Naltrexol	1.3

Analyte recovery from the Cyclone-MAX column was assessed via analysis of an aqueous solution (all analytes 100 µg/L) through a) the complete TurboFlow process, and b) with the TurboFlow system bypassed (i.e. injection directly onto the analytical column). The mean peak areas (N = 3) of each analyte subjected to the complete TurboFlow process were compared to those observed from injection onto the analytical column only, in which the latter was assumed to represent 100 % recovery. Good recovery for all analytes from the Cyclone-MAX column was observed using these conditions with the exception of nornaloxone (Table 7.11a). Poor recovery of nornaloxone was attributed to loss in the loading step. To improve the retention of nornaloxone, a Cyclone-P TurboFlow column was added in tandem after the Cyclone-MAX column to retain nornaloxone (Figure 7.6). Through inclusion of this second TurboFlow column, recovery of nornaloxone was improved (Table 7.11b).

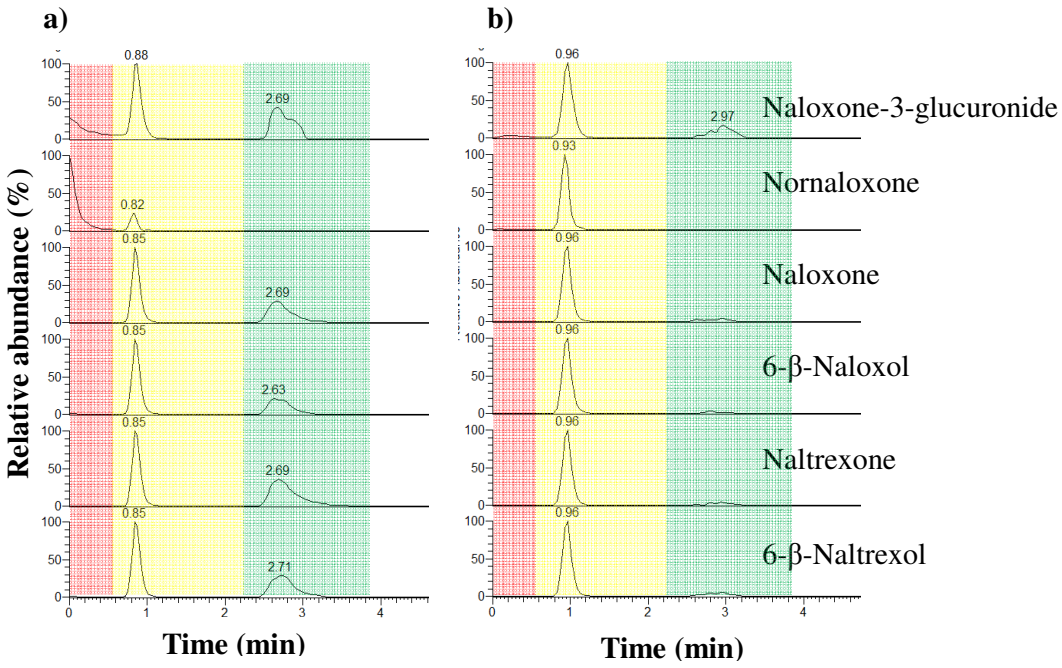
Table 7.11 – Naloxone/naltrexone assay: Recovery from a) Cyclone-MAX TurboFlow column, and b) Cyclone-MAX and Cyclone-P TurboFlow columns in tandem

Recovery was assessed through peak area comparison of an aqueous solution containing all analytes (100 µg/L) injected onto i) the TurboFlow column/s (N=3), and ii) directly injected on the analytical column (N=3).

Analyte	TurboFlow Recovery (%)	
	a) Cyclone MAX Only	b) Cyclone MAX & Cyclone-P
Naloxone	94	89
Naloxone-D_5	88	89
Nornaloxone	15	65
6-β-Naloxol	85	91
Naloxone-3-glucuronide	76	84
Naltrexone	96	91
Naltrexone-D_4	94	91
6-β-Naltrexol	78	93

<u>Figure 7.6</u> – Naloxone/naltrexone assay: Retention of analytes on a) Cyclone-MAX TurboFlow column, and b) Cyclone-MAX and Cyclone-P TurboFlow columns in tandem

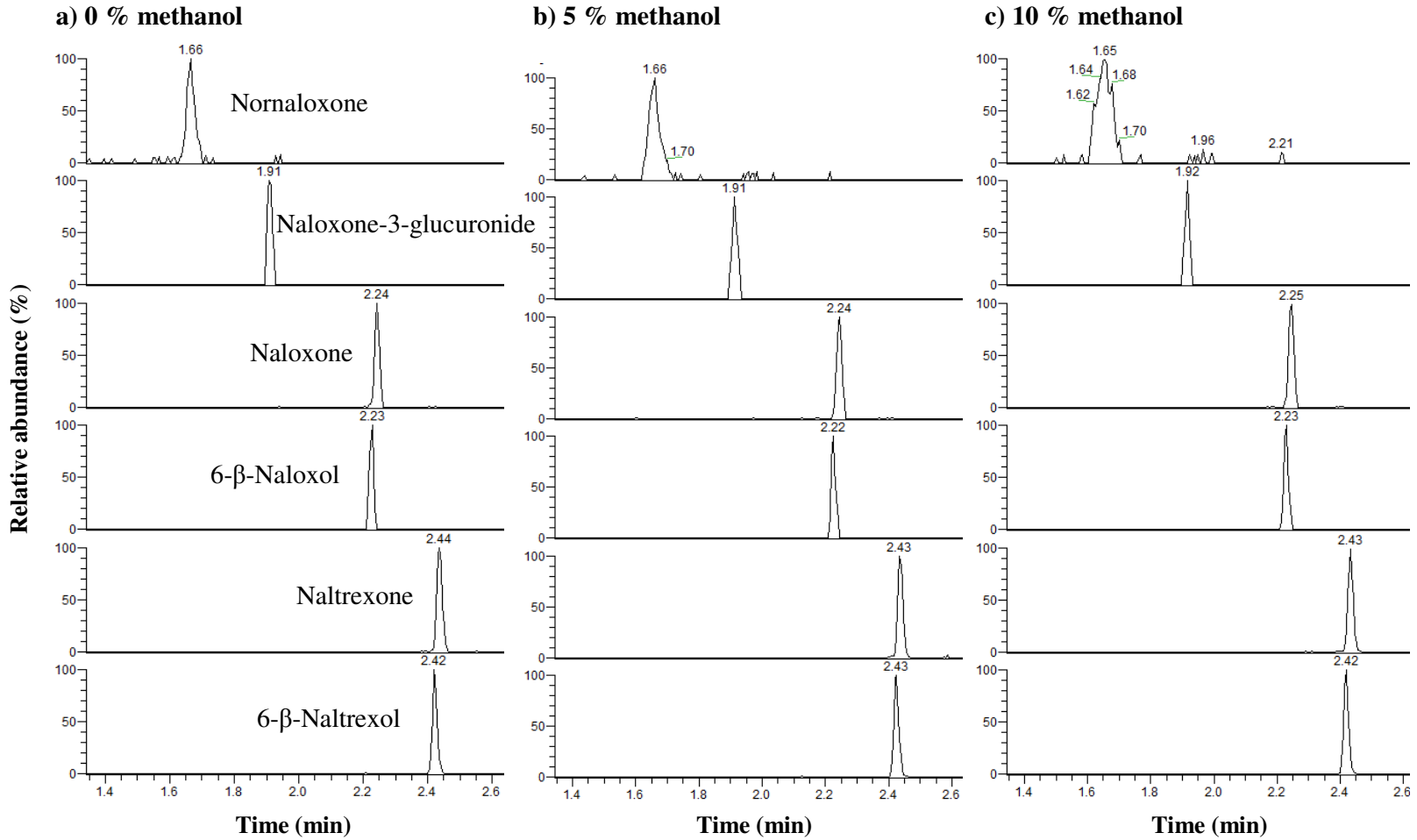
The loading, transfer, and eluting steps are represented by the red, yellow, and green shaded areas respectively. For maximum analyte recovery, peaks should appear only in the transfer step.



Due to the change in LC method first from the Hypercarb to the Biphenyl column, and subsequently the change from ammonium bicarbonate to aqueous formic acid eluent, compatibility of the TurboFlow method with the LC method was reassessed. To ascertain the maximum organic content of the injection solvent possible without impacting nornaloxone retention, solutions containing all analytes (0.05 µg/L) from 0-50 % organic content (in 5 % increments) were analysed using the LC method, i.e. bypassing the TurboFlow column (Figure 7.7).

The retention of nornaloxone was affected when 5 % methanol was present in the injection solvent, thus ideally the injection solvent should contain no organic solvent. On this basis, using TurboFlow would not be possible as organic solvent is introduced during the transfer step. Liquid-liquid extraction and protein precipitation were thus investigated as alternative sample preparation methods.

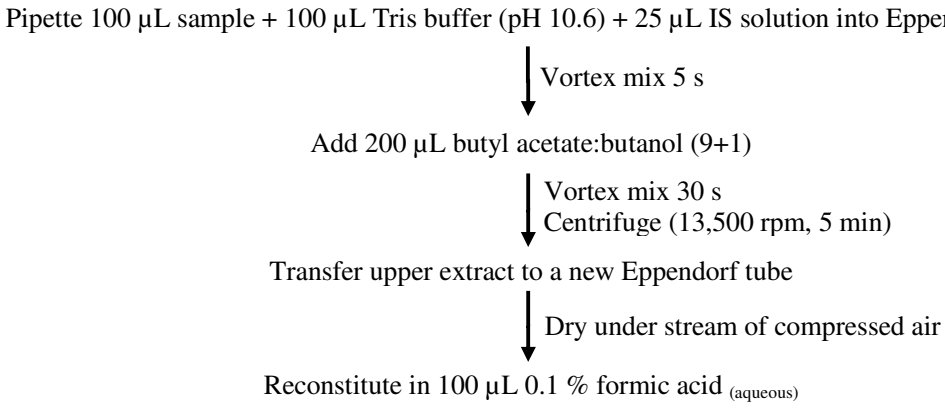
Figure 7.7 – Naloxone/naltrexone assay: The influence of increasing the proportion of methanol (%) in the injection solvent on the retention of nornaloxone [Biphenyl (100 x 2.1 I.D. mm) column, Restek]



7.3.3.2 Liquid-Liquid Extraction

A simple LLE protocol (Figure 7.8) was used for initial investigations. The median (range) *p*Ka for the analytes was 8.6 (7.8-9.5). For optimum analyte extraction the pH should be 2 units above analyte *p*Ka, thus pH 10.6 was initially tried.

Figure 7.8 – Naloxone/naltrexone assay: LLE protocol



To access the effect of pH on extraction efficiency, the aqueous phase pH was altered over the range 5-10.5 (in 0.5 pH unit increments). The same protocol was used, but with the buffer varied (2 mol/L Tris pH 7.5-10.5, 2 mol/L ammonium acetate pH 5-7). Naloxone-3-glucuronide and nornaloxone exhibited poor extraction, with naloxone-3-glucuronide not extracted at any pH and the best recovery for nornaloxone reaching only 8 %. For the other analytes, pH 9 gave the best recovery (Table 7.12).

Table 7.12 – Naloxone/naltrexone assay: Recovery of analytes under different pH conditions using LLE

Analyte	Analyte recovery at varying pH (%)											
	5	**5.5**	**6**	**6.5**	**7**	**7.5**	**8**	**8.5**	**9**	**9.5**	**10**	**10.5**
Naloxone-3-glucuronide	0	0	0	0	0	0	0	0	0	0	0	0
Nornaloxone	0	0	0	0	0	1	3	5	7	8	7	3
Naloxone	12	26	48	62	74	83	82	80	86	83	78	79
6-β-Naloxol	1	2	5	13	25	59	62	64	71	68	61	58
Naltrexone	4	6	24	44	62	87	91	87	94	92	84	88
6-β-Naltrexol	0	1	2	5	12	46	58	62	75	75	67	72

'Salting out' has been shown to encourage some more polar analytes to move into the organic phase during LLE (Flanagan *et al.*, 2006; Kole *et al.*, 2011). To access whether 'salting out' might improve the recovery of naloxone-3-glucuronide and nornaloxone, 50 µL of saturated NaCl solution was added at the initial pipetting stage. Results showed no improvement. Whilst LLE gave good results for most analytes, the more polar analytes were not extracted, and thus LLE was deemed unsuitable for the assay.

7.3.3.3 Protein Precipitation

Protein precipitation is non-selective which may be beneficial when analytes possess different physicochemical properties; however more matrix interferences are likely to remain which may affect analyte quantitation. Two of the most effective precipitants for protein removal are acetonitrile and aqueous trichloroacetic acid (TCA) (Polson *et al.*, 2003). TCA precipitation was investigated first as addition would negate the need for a drying down step. 50 µL aqueous TCA (10 % w/v) were added to 200 µL plasma sample (standard 1, see Table 7.5) in an Eppendorf tube. The contents of the tube were vortex-mixed (30 s) and then centrifuged (5 min, 13,500 rpm). The upper layer was transferred to a HPLC vial and 100 µL of extract were injected. Results showed that the sample dilution associated with TCA precipitation was unacceptable, with some analytes not being detected in the lowest calibrator. Whilst protein precipitation using acetonitrile necessitates a drying down step, and hence is a lengthier sample preparation procedure, it also facilitates sample concentration. A basic protocol (Figure 7.9) was thus used for initial investigations using acetonitrile precipitation.

To ascertain the optimum ratio of sample to solvent, the volume of IS solution added was varied. A balance between improved protein removal and additional drying time needed to be assessed. Optimum results were observed using a ratio of 1+3 sample:solvent (IS solution). To reduce drying time, the time to dry samples in a heating block at 40 ^{o}C was compared to the time to dry samples at ambient temperature. No analyte degradation was noted and drying time was reduced, thus drying at 40 ºC was included in the protocol. To improve assay sensitivity, sample concentration during the reconstitution step was evaluated. Optimum results were observed when the sample was concentrated by a factor of 2. To ascertain the minimum mixing time required for complete protein precipitation the procedure was assessed over the range of 0.5-10 min. No benefit was observed when mixing was longer than 1 min, thus this was set as the optimum time in the final method (Figure 7.10). Analysis of the lowest calibration solution using this protocol gave adequate sensitivity (Figure 7.11)

Figure 7.9 – Initial protein precipitation protocol using acetonitrile

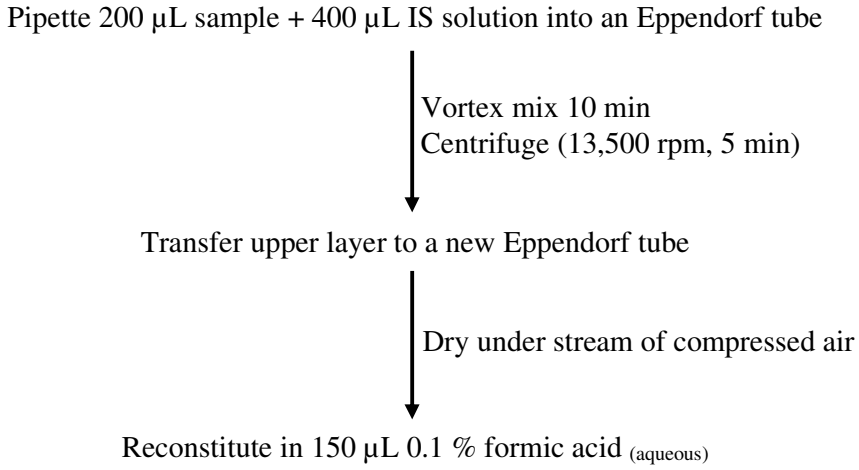


Figure 7.10 – Naloxone/naltrexone assay: Final protein precipitation protocol

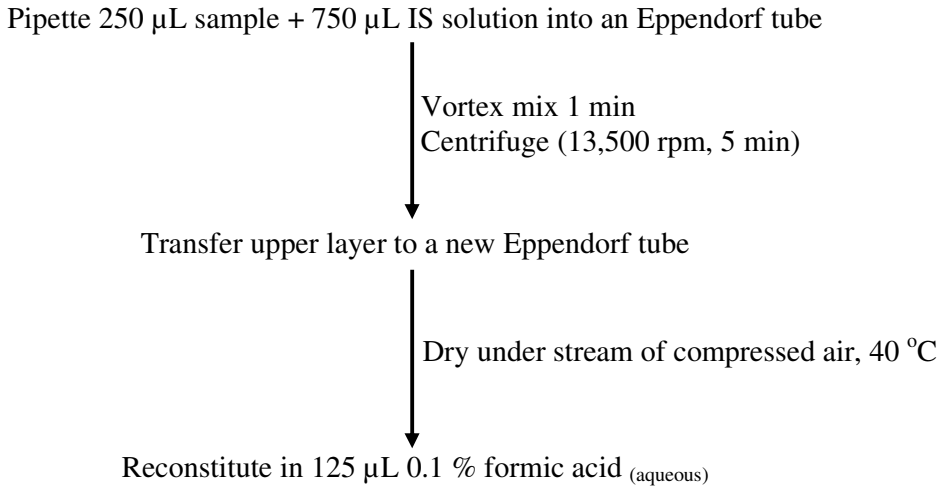
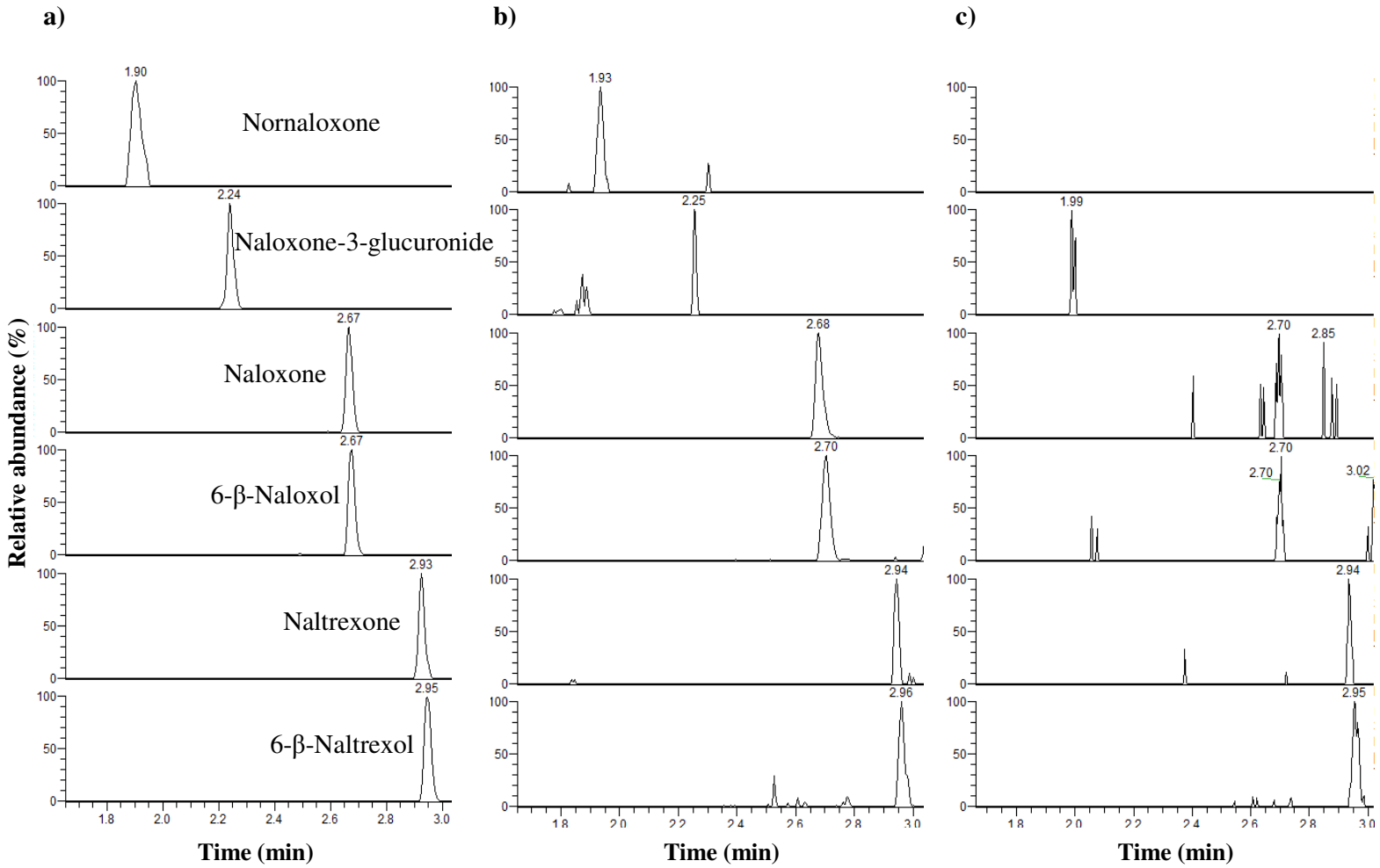


Figure 7.11 – Comparison of protein precipitation techniques for naloxone/naltrexone assay: Injection of a) an aqueous solution (comparable to standard 1) was compared to standard 1 prepared by protein precipitation using b) acetonitrile, and c) TCA



7.3.4 Assay Calibration

7.3.4.1 Selection of a suitable calibration range

Reported peak plasma concentrations via different routes of administration are given in Table 7.13. There are minimal data regarding the concentration of naloxone metabolites in plasma after administration of naloxone. Plasma nornaloxone concentrations of less than 0.5 µg/L were reported for 3 individuals maintained on sublingual 16 mg buprenorphine/4 mg naloxone for 2 weeks (Fang *et al.*, 2009).

The literature suggests that steady state plasma naltrexone and 6-β-naltrexol concentrations are in the range of 3-25 µg/L and 25-70 µg/L, respectively (Brünen *et al.*, 2010; Ferrari *et al.*, 1998).

Table 7.13 – Reported plasma naloxone PK parameters via differing administration routes

[a] bolus followed by infusion (0.24 mg/min for 5 h)
* co-formulated with buprenorphine (dose ratio 4:1 buprenorphine:naloxone)

Study	N	Route of administration	Dose (mg)	C_{max} (µg/L)	T_{max} (min)
Krieter *et al.*, 2016	29	Intramuscular	0.4	0.9	24
Baselt, 2014	6	Intramuscular	0.8	1.4	15
Krieter *et al.*, 2016	29	Intranasal	2	3.1	18
Krieter *et al.*, 2016	29	Intranasal	4	5.3	18
Krieter *et al.*, 2016	29	Intranasal	8	10.3	18
Baselt, 2014	6	Intranasal	2	0.8	10
Baselt, 2014	9	Intravenous	0.4	10	2
Reid *et al.*, 1993	2	Intravenous	20[a]	69-84	-
Baselt, 2014	20	Oral	5	0.032	300
Baselt, 2014	20	Oral	20	0.099	300
Moody *et al.*, 2002	3	Sublingual*	2	0-0.2	30
Fang *et al.*, 2009	3	Sublingual*	4	0.139 ±0.062	30
Chiang & Hawks, 2003	9	Sublingual*	4	0.66 ±0.53	56 ±15
Chiang & Hawks, 2003	9	Sublingual*	8	0.93 ±0.71	61 ±19

7.3.4.2 Internal Standards

Initially deuterated analogues of each parent drug, naloxone-D_5 and naltrexone-D_4, were selected as internal standards.

Naltrexone-D_4 was later replaced by naltrexone-D_7 due to interference from a naturally occurring isotope of 6-β-naltrexol in the analysis. The second natural isotope (M+2) of 6-β-naltrexol (*m/z* 346.1910) is close in mass to naltrexone-D_4 (*m/z* 346.1951). These two masses could not be completely resolved even using high resolution MS (Figure 7.12). As a result, the peak area of naltrexone-D_4 was falsely elevated as the 6-β-naltrexol concentration increased. This did not occur when naltrexone-D_7 was used as the internal standard, with the naltrexone-D_7 peak area remaining consistent between calibrators and IQCs (Figure 7.13).

Naloxone-3-glucuronide was subject to a far greater matrix effect than the naloxone-D_5 which was being used as an internal standard. To resolve the issue, naloxone-3-glucuronide-D_5 was added to the method (see Section 7.3.5).

<u>Figure 7.12</u> – Mass chromatograms to show the effect of increasing resolution to attempt to remove 6-β-naltrexol interference on naltrexone-D_4

The 10 ppm extraction window based upon naltrexone-D_4 (*m/z* 346.1951) is represented by the shaded box.

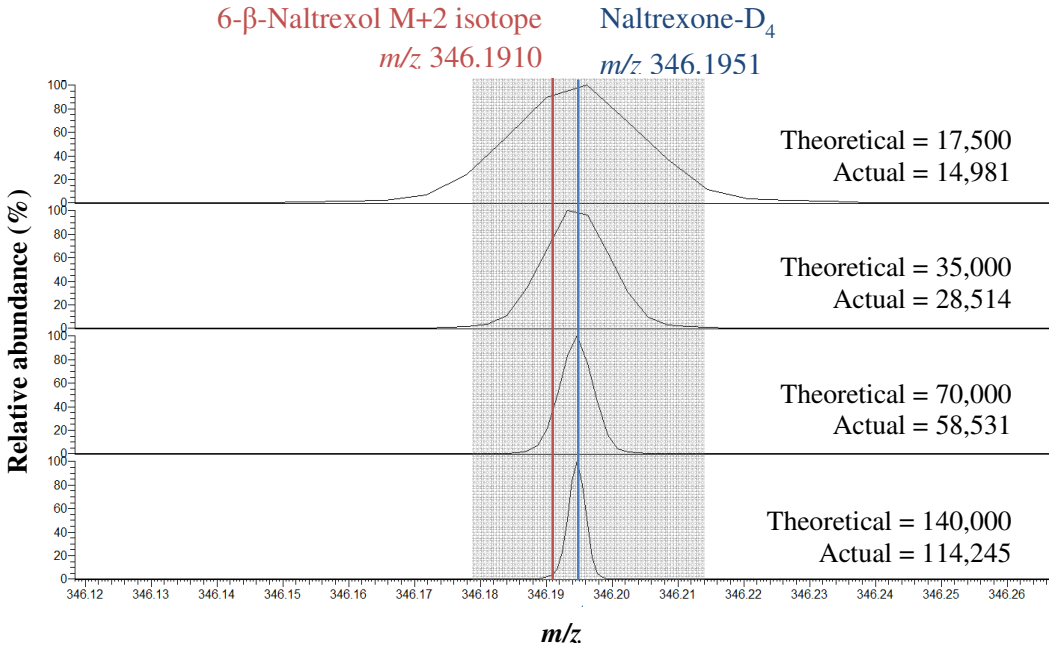
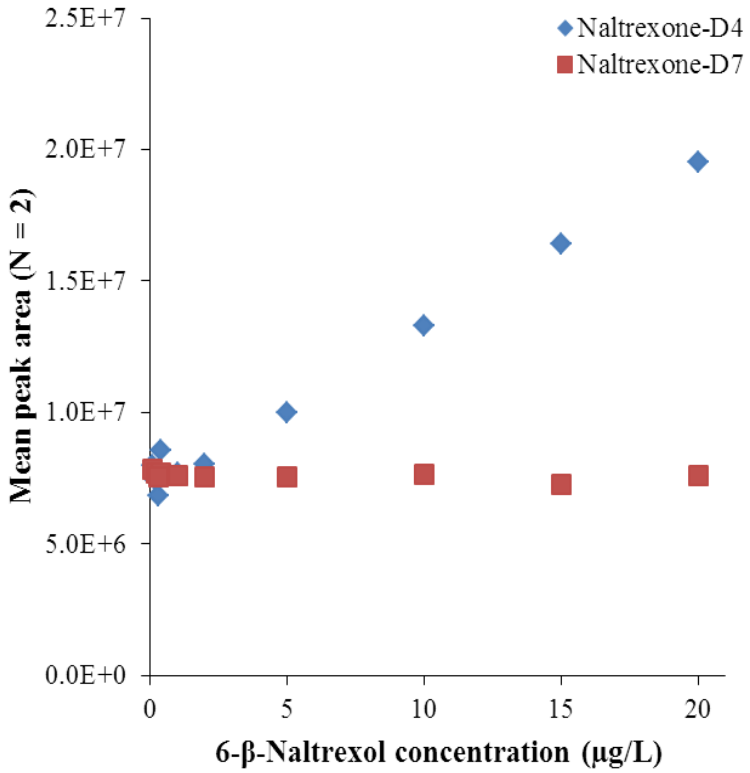


Figure 7.13 – The effect of 6-β-naltrexol concentration on naltrexone-D_4 peak area

Mean peak areas (N = 2) for (i) naltrexone-D_4, and (ii) naltrexone-D_7 were compared in calibrator and IQC samples.



7.3.5 Assay Validation

Intra- and inter-assay accuracy and precision are summarized in Tables 7.14 and 7.15, respectively. The limit of accurate measurement was taken as the lowest calibrator, with good precision observed for all analytes in both urine and plasma (RSD <10 %). Calibration graphs were linear (R^2 ≥0.99 for all analytes) over the calibration ranges (Figures 7.14 and 7.15). Linearity was good for naloxone, naloxone-3-glucuronide, and naltrexone above the top calibrator in both urine and plasma (Figure 7.16). However, the accuracy of the measured concentration of 6-β-naloxol, 6-β-naltrexol, and nornaloxone deteriorated at higher concentration indicating that reanalysis after sample dilution is required for accurate quantitation of these analytes. Analyte recovery from plasma was good (Table 7.16). No significant carryover/carryunder was observed in plasma or urine samples (Table 7.17). Ion suppression was identified (Figure 7.17), but the internal standards used compensated well for matrix effects, except that nornaloxone showed some variability in plasma and whole blood samples (Table 7.18).

Table 7.14 – Plasma naloxone/naltrexone assay: Summary intra- and inter-assay accuracy and precision data

Analyte	Nominal concentration (µg/L)	Mean measured concentration (µg/L)	Accuracy (% nominal)	RSD (%)
a) Intra-assay				
Naloxone	0.15	0.14	95	6
	2.50	2.33	93	4
	7.50	7.20	96	3
Nornaloxone	0.30	0.31	102	5
	5.00	4.24	85	4
	15.0	14.1	94	5
6-β-Naloxol	0.15	0.15	100	12
	2.50	2.70	108	5
	7.50	7.93	106	4
Naloxone-3-glucuronide	1.50	1.56	104	5
	25.0	24.0	96	9
	75.0	77.5	103	8
Naltrexone	0.30	0.32	105	3
	5.00	5.11	102	5
	15.0	14.7	98	2
6-β-Naltrexol	0.30	0.35	117	14
	5.00	5.85	117	10
	15.0	15.3	102	3
b) Inter-assay				
Naloxone	0.15	0.14	94	8
	2.50	2.42	97	6
	7.50	7.06	94	6
Nornaloxone	0.30	0.28	94	5
	5.00	4.50	90	4
	15.0	13.7	91	6
6-β-Naloxol	0.15	0.14	95	9
	2.50	2.64	106	4
	7.50	7.20	96	6
Naloxone-3-glucuronide	1.50	1.50	100	14
	25.0	23.7	95	4
	75.0	70.8	94	3
Naltrexone	0.30	0.29	98	3
	5.00	5.05	101	4
	15.0	14.2	95	2
6-β-Naltrexol	0.30	0.32	105	12
	5.00	5.62	112	12
	15.0	13.9	93	6

Table 7.15 – Urine naloxone/naltrexone assay: Summary intra- and inter-assay accuracy and precision data

Analyte	Nominal concentration (µg/L)	Mean measured concentration (µg/L)	Accuracy (% nominal)	RSD (%)
a) Intra-assay				
Naloxone	20	20.8	104	3
	300	338.3	113	2
	550	487.4	89	3
Nornaloxone	20	18.7	93	2
	300	293.3	98	3
	550	468.6	85	3
6-β-Naloxol	20	21.5	107	2
	300	334.6	112	2
	550	482.7	88	2
Naloxone-3-glucuronide	20	17.2	86	2
	300	308.0	103	2
	550	476.4	87	2
Naltrexone	20	20.1	100	2
	300	321.9	107	2
	550	479.3	87	2
6-β-Naltrexol	20	16.9	84	2
	300	339.4	113	2
	550	484.7	88	2
b) Inter-assay				
Naloxone	20	21.8	109	4
	300	335.9	112	3
	550	500.9	91	3
Nornaloxone	20	19.4	97	7
	300	302.8	101	7
	550	487.2	89	3
6-β-Naloxol	20	23.7	118	8
	300	341.6	114	3
	550	491.7	89	3
Naloxone-3-glucuronide	20	18.3	91	9
	300	308.3	103	3
	550	493.3	90	4
Naltrexone	20	21.4	107	9
	300	314.3	105	7
	550	496.3	90	3
6-β-Naltrexol	20	16.9	85	10
	300	326.5	109	10
	550	498.9	91	4

Figure 7.14 – Naloxone/naltrexone assay: Typical calibration curves for all analytes in plasma

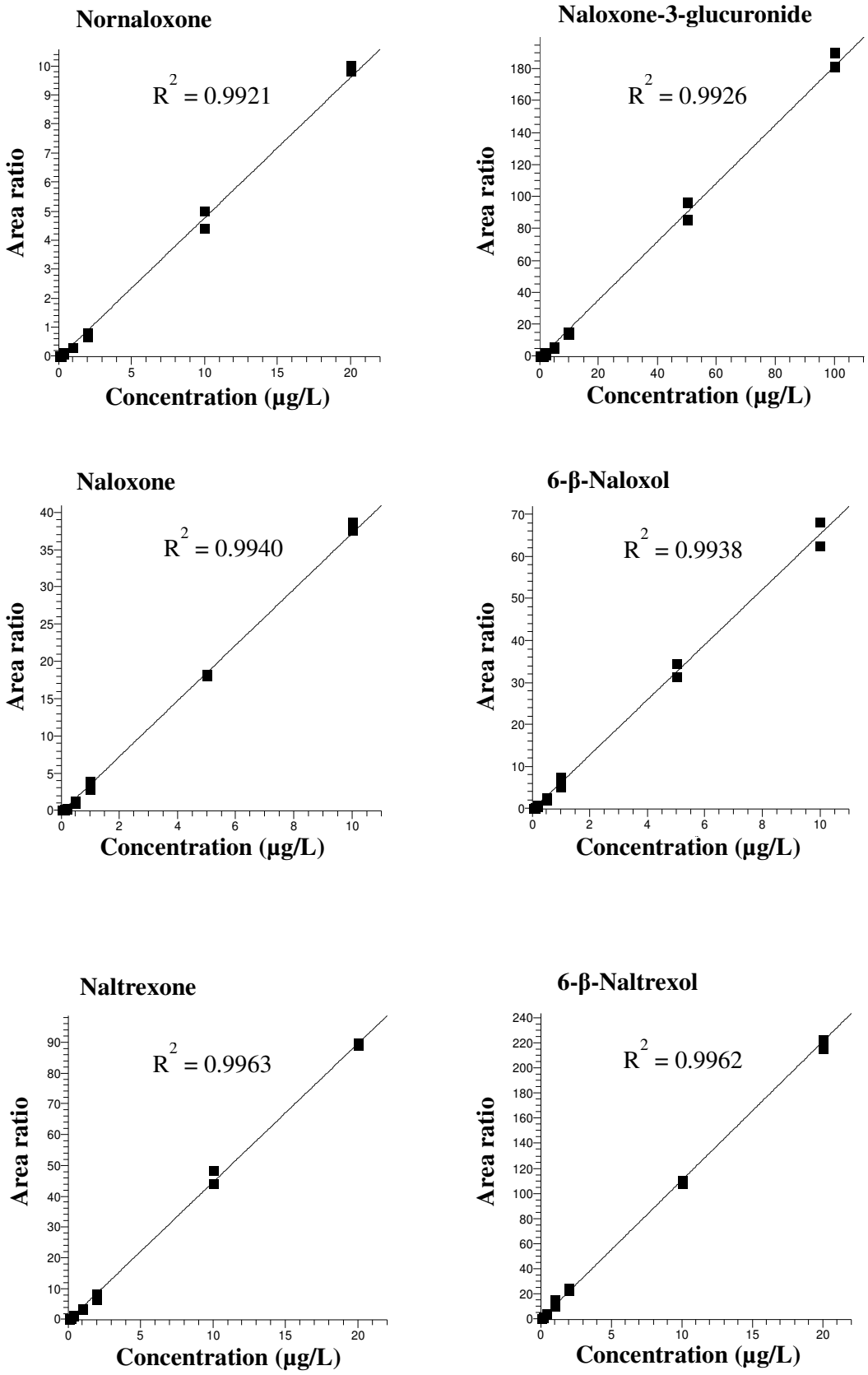


Figure 7.15 – Naloxone/naltrexone assay: Typical calibration curves for all analytes in urine

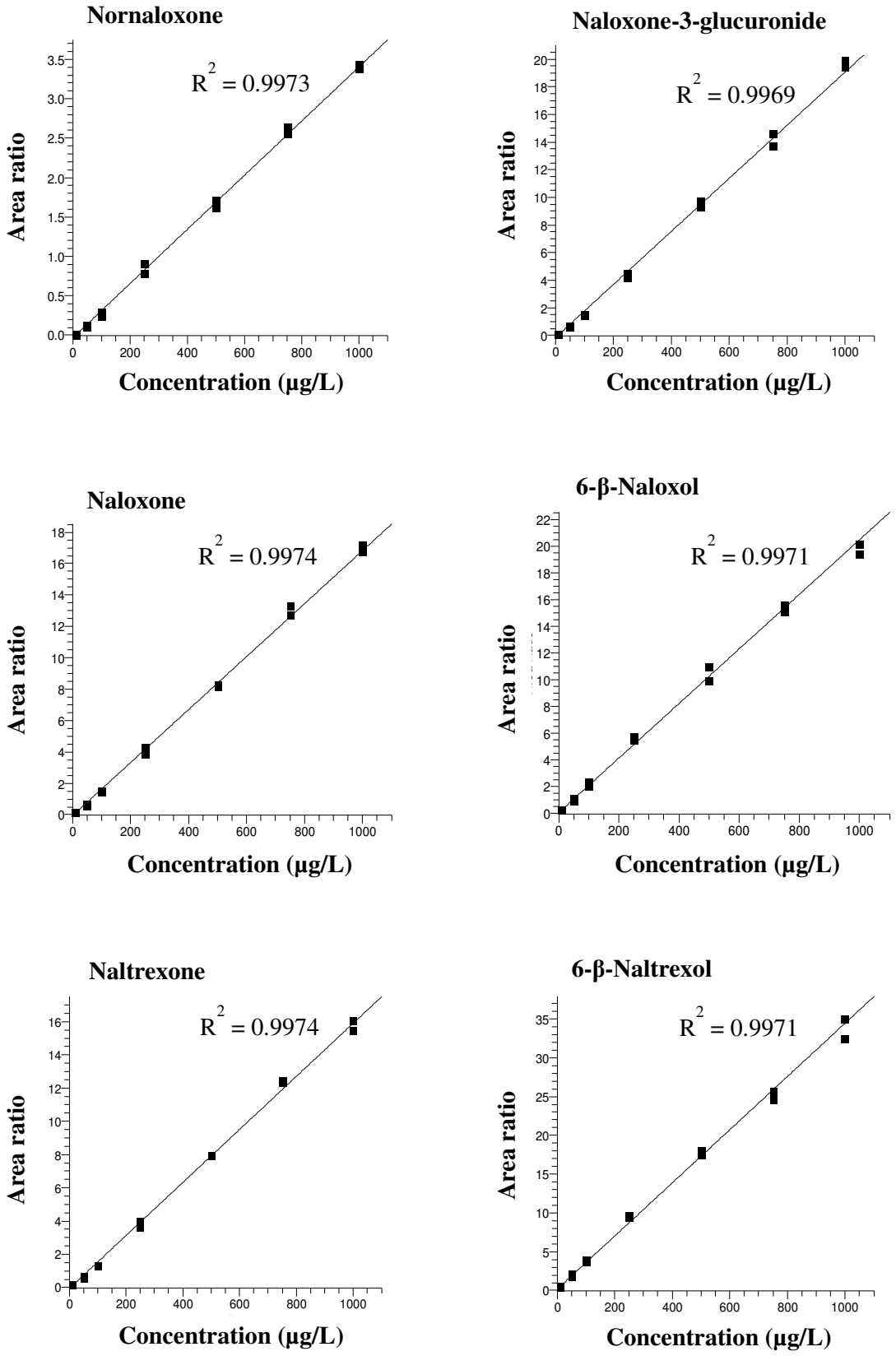
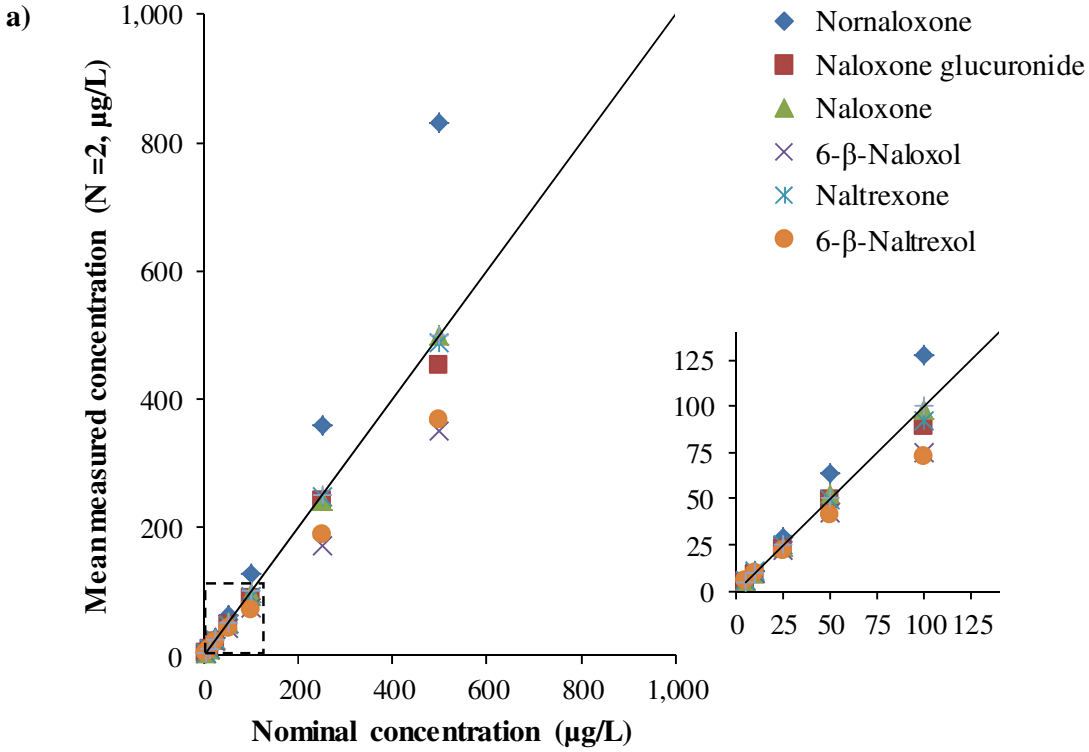


Figure 7.16 – Naloxone/naltrexone assay: Analyte linearity for a) plasma, and b) urine



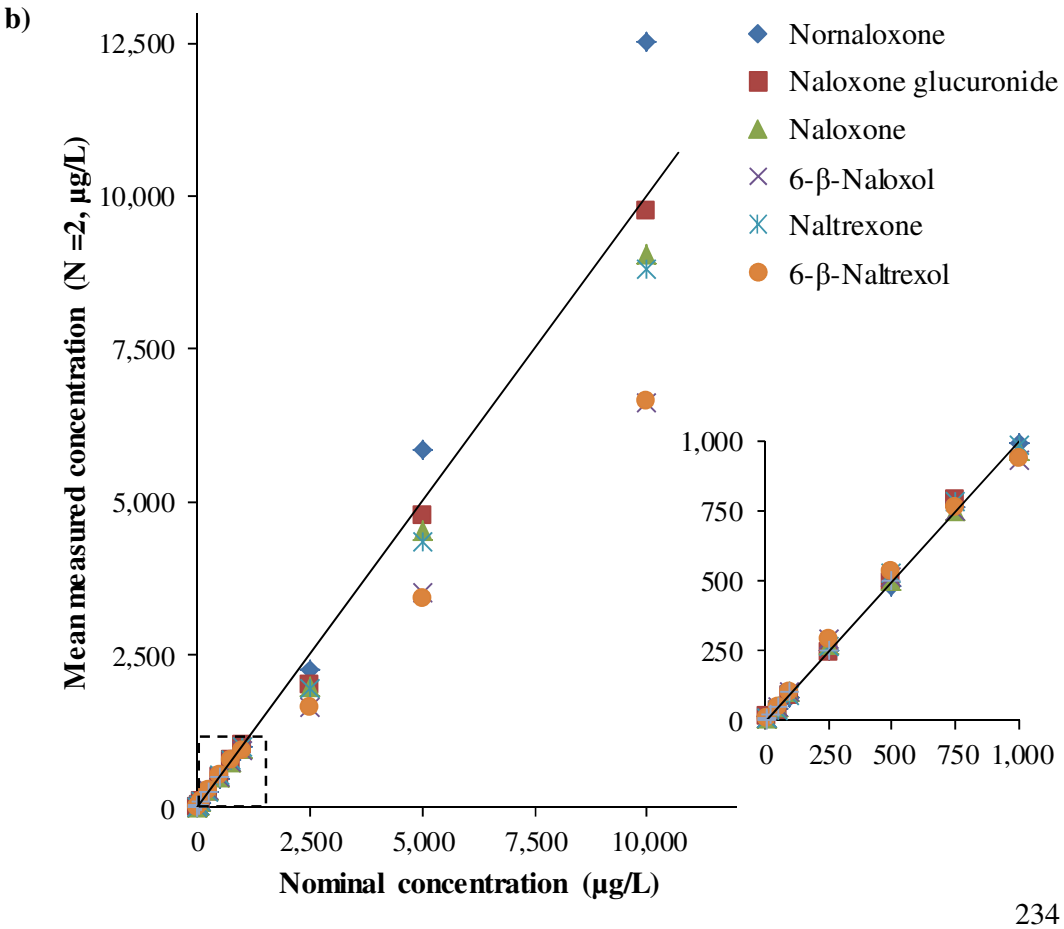


Table 7.16 – Naloxone/naltrexone assay: Analyte recovery from plasma

Analyte	No extraction mean peak area	Post extraction mean peak area	Recovery (%)
Naloxone	1.13 e^8	1.03 e^8	91
Nornaloxone	3.91 e^8	3.42 e^7	87
6-β-Naloxol	1.47 e^8	1.34 e^8	91
Naloxone-3-glucuronide	2.36 e^7	2.25 e^7	96
Naltrexone	1.19 e^8	1.13 e^8	95
6-β-Naltrexol	2.39 e^8	2.16 e^8	90
Naloxone-D_5	9.39 e^7	8.63 e^7	92
Naloxone-3-glucuronide-D_5	1.88 e^7	1.78 e^7	95
Naltrexone-D_7	9.97 e^7	9.52 e^7	95

Table 7.17 – Naloxone/naltrexone assay: Analyte carryover in plasma and urine

Carryover was assessed through comparison of the measured concentration of a low concentration sample (L) analysed after a high concentration sample (H) to the mean measured concentration of the low concentration sample analysed prior to the high concentration sample (N = 3).

Plasma: L = all analytes 0.5 µg/L, H = all analytes 50 µg/L

Urine: L = all analytes 10 µg/L, H = all analytes 2000 µg/L

Analyte	Carryover (%)	
	Plasma	Urine
Naloxone	0	0
Nornaloxone	6	-1
6-β-Naloxol	5	-9
Naloxone-3-glucuronide	-3	1
Naltrexone	3	-5
6-β-Naltrexol	4	-2

Figure 7.17 – Naloxone/naltrexone assay: Post-column infusion of analytes to qualitatively assess matrix effects in human plasma. Extracted ion chromatograms for each analyte to show a) the solvent blank (black trace), b) matrix blank (red trace), and c) analyte retention time (green trace)

Ion suppression is identified where the trace for the matrix blank is lower than the trace for the solvent blank.

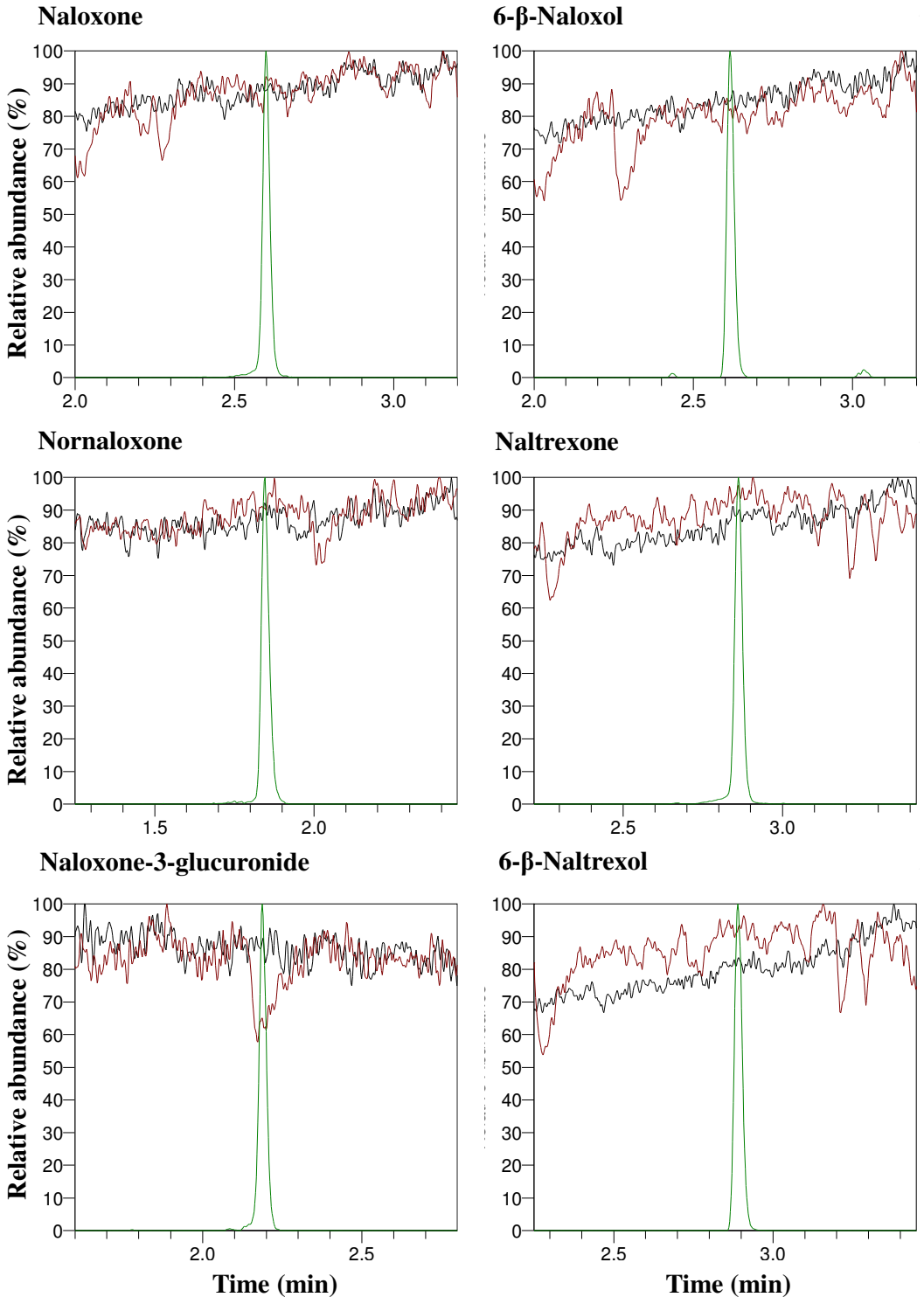


Table 7.18 - Naloxone/naltrexone assay: Observed matrix effects in a) plasma, b) whole blood, and c) urine

Matrix effect (%) is defined as the ratio of the analyte peak area in the presence of matrix to that in the absence of matrix. The relative matrix effect (%) was calculated through comparison of the analyte matrix effect with that of its internal standard.

Analyte	Peak area in the absence of matrix	Peak area in the presence of matrix	Matrix effect (%)	Relative matrix effect (%)
a) Plasma				
Naloxone	1.24 e^8	7.98 e^7	65	112
Nornaloxone	4.04 e^7	3.08 e^7	76	132
6-β-Naloxol	1.33 e^8	8.81 e^7	66	115
Naloxone-3-glucuronide	2.15 e^7	7.53 e^6	35	101
Naltrexone	1.29 e^8	7.36 e^7	57	105
6-β-Naltrexol	2.00 e^8	1.11 e^8	56	102
Naloxone-D_5	1.10 e^7	6.32 e^7	58	-
Naloxone-3-glucuronide-D_5	1.23 e^7	4.24 e^6	35	-
Naltrexone-D_7	9.16 e^7	5.01 e^6	55	-
b) Whole blood				
Naloxone	4.95 e^8	3.93 e^8	79	98
Nornaloxone	2.52 e^8	9.88 e^7	39	48
6-β-Naloxol	5.40 e^8	3.84 e^8	71	88
Naloxone-3-glucuronide	1.93 e^8	1.71 e^7	9	104
Naltrexone	5.10 e^8	3.75 e^8	73	110
6-β-Naltrexol	8.02 e^8	5.56 e^8	69	103
Naloxone-D_5	6.24 e^8	5.05 e^8	81	-
Naloxone-3-glucuronide-D_5	2.11 e^8	1.80 e^7	9	-
Naltrexone-D_7	6.01 e^8	4.03 e^8	67	-
c) Urine				
Naloxone	2.65 e^7	2.10 e^7	79	107
Nornaloxone	7.59 e^6	5.29 e^6	70	94
6-β-Naloxol	3.82 e^7	2.40 e^7	63	85
Naloxone-3-glucuronide	4.26 e^6	3.71 e^6	87	108
Naltrexone	2.74 e^7	2.12 e^7	77	104
6-β-Naltrexol	6.10 e^7	4.18 e^7	69	92
Naloxone-D_5	5.14 e^7	3.80 e^7	74	-
Naloxone-3-glucuronide-D_5	7.75 e^6	6.30 e^6	81	-
Naltrexone-D_7	6.28 e^7	4.68 e^7	74	-

7.3.5.1 Patient Samples

A summary of the samples analysed to clinically validate the developed assay and demonstrate it was fit-for-purpose are given in Tables 7.19 and 7.20. The dose of drug and the time since drug administration were unknown.

Table 7.19 – Analyte concentration in urine: summary of naloxone, naltrexone, and their metabolites in urine from patients administered a) naloxone, b) Suboxone, and c) naltrexone

*cases of Suboxone adulteration excluded (N = 7)

Drug administered	N	Concentration (µg/L)			
		Mean	Median	Min	Max
Naloxone	18				
Naloxone	8	33	14	11	114
Nornaloxone	2	33	33	14	51
6-β-Naloxol	5	21	17	15	35
Naloxone-3-glucuronide	18	720	418	92	2,370
Suboxone*	11				
Naloxone	3	23	24	15	30
Nornaloxone	4	22	19	12	37
6-β-Naloxol	1	20	-	-	-
Naloxone-3-glucuronide	11	968	405	29	6,310
Naltrexone	6				
Naltrexone	6	237	172	28	736
6-β-Naltrexol	6	19,000	20,000	4,540	35,400

Table 7.20 – Analyte concentration in plasma: summary of naloxone, naltrexone, and their metabolites in plasma from patients administered a) naloxone, and b) naltrexone

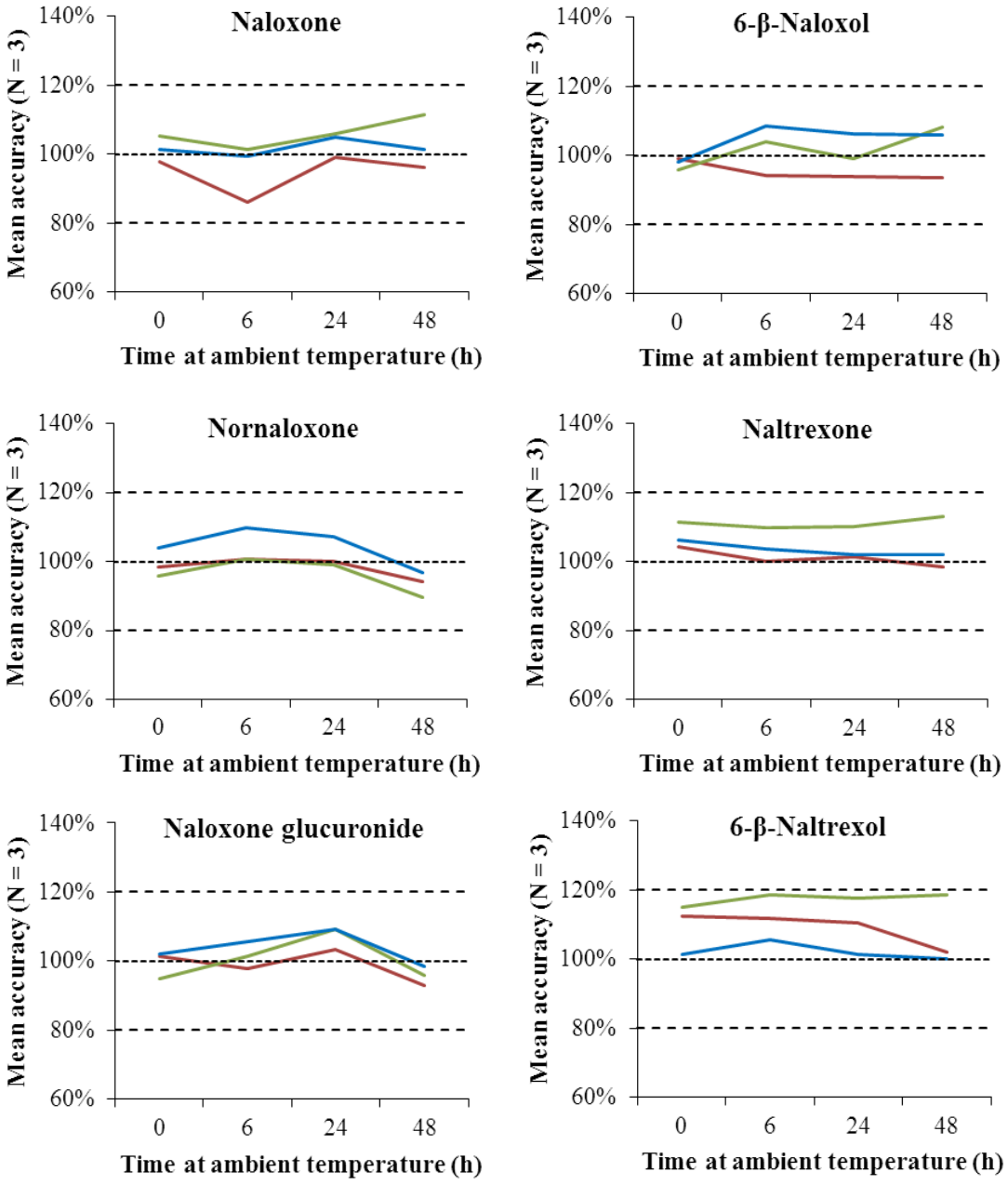
Drug administered	N	Concentration (µg/L)			
		Mean	Median	Min	Max
Naloxone	5				
Naloxone	5	1.29	0.45	0.07	2.89
Nornaloxone	2	0.13	0.13	0.11	0.15
6-β-Naloxol	5	0.45	0.33	0.16	1.06
Naloxone-3-glucuronide	5	9.65	6.59	1.24	30.6
Naltrexone	1				
Naltrexone	1	0.12	-	-	-
6-β-Naltrexol	1	12.0	-	-	-

7.3.6 Analyte Stability in Human Plasma

7.3.6.1 Ambient Temperature

IQC solutions (N = 3) stored at room temperature were analysed over a 48 h period. The median (range) ambient temperature over this time period was 18.7 (15.3-23.0) ^{o}C. The results showed that all analytes were stable for at least 48 h at room temperature (Figure 7.18).

Figure 7.18 – Naloxone/naltrexone assay: Analyte ambient temperature stability
Mean accuracy calculated through comparison of mean measured concentration (N = 3) to the target IQC concentration. Acceptable accuracy is delimited by dashed lines, and IQC A, B and C represented by red, green, and blue lines respectively.

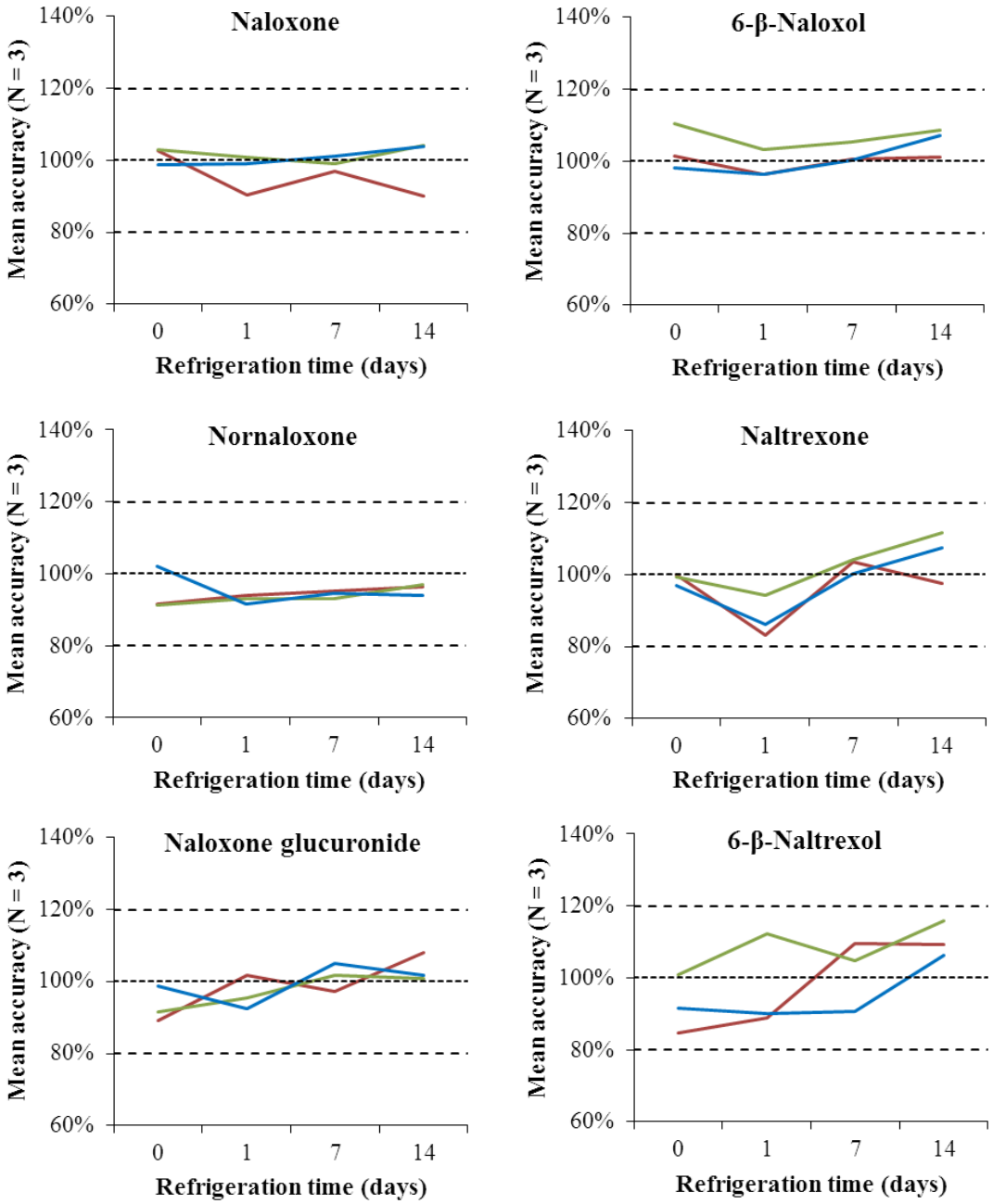


7.3.6.2 Refrigerator Temperature

IQC solutions (N = 3) stored refrigerated were analysed over a 2-week period. The median (range) temperature over this time period was 5.4 (1.5-9.9) ^{o}C. The results showed that all analytes are stable for at least 2 weeks (Figure 7.19).

Figure 7.19 – Naloxone/naltrexone assay: Analyte refrigerator stability

Mean accuracy calculated through comparison of mean measured concentration (N = 3) to the target IQC concentration. Acceptable accuracy is delimited by dashed lines, and IQC A, B and C represented by red, green, and blue lines respectively.

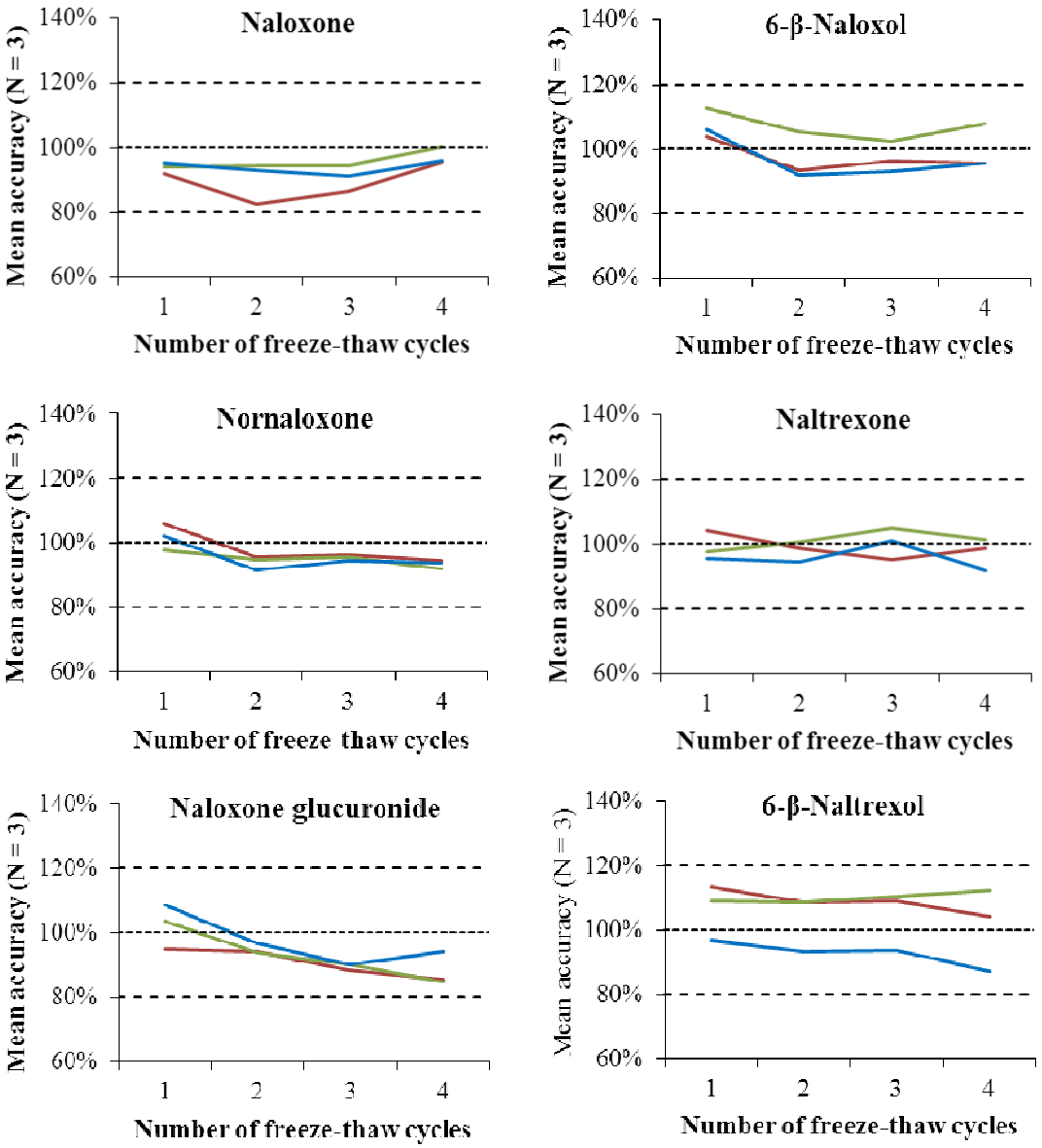


7.3.6.3 Freeze-thaw stability

IQC solutions (N = 3) were analysed after 4 successive freeze-thaw cycles. The median (range) freezer temperature over this time period was -26.7 (-17.4 to -28.2) ^{o}C. IQC solutions were thawed by inversion mixing at room temperature prior to each analysis. The samples were returned to freezer storage conditions for at least 24 h prior to removal for the next freeze thaw cycle. All analytes were stable following 4 freeze-thaw cycles (Figure 7.20).

Figure 7.20 – Naloxone/naltrexone assay: Analyte freeze-thaw stability

Mean accuracy calculated through comparison of mean measured concentration (N = 3) to the target IQC concentration. Acceptable accuracy is delimited by dashed lines, and IQC A, B and C represented by red, green, and blue lines respectively.

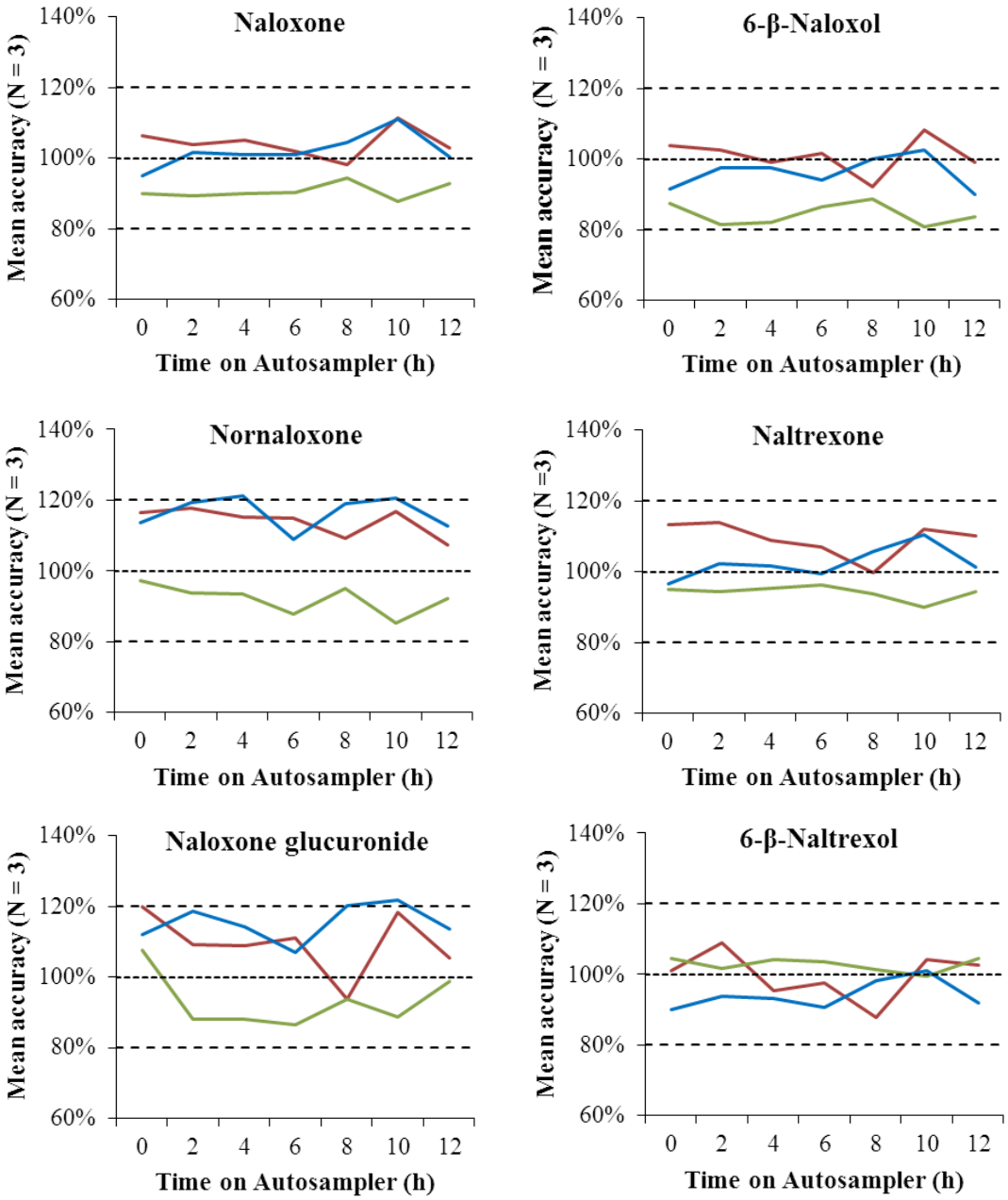


7.3.6.4 Extract Stability

Separate vials of prepared standard solutions (N = 3) were stored at 4 °C on the autosampler and analysed every 2 hours over a 12 h period. Data showed all analytes are stable on the autosampler for at least 12 h (Figure 7.21). The maximum number of patient sample which can be analysed in a batch within this timeframe is 90 samples.

Figure 7.21 – Naloxone/naltrexone assay: Analyte autosampler stability

Accuracy calculated through comparison of measured concentration to the target standard concentration. Acceptable accuracy is delimited by dashed lines, and Standards 2, 4 and 7 represented by red, green, and blue lines respectively.



7.3.7 Differentiation of Suboxone and Subutex Use

Naloxone-3-glucuronide was detected in 12 of the 14 urine samples collected from individuals known to be taking Suboxone. The median (range) naloxone-3-glucuronide concentration was 754 (39-6,150) µg/L. 6-β-Naloxol was detected in one sample (41 µg/L). Naloxone and nornaloxone were not detected in any of the samples. Analysis of urine samples from individuals prescribed Subutex had no naloxone or metabolites detected.

In both samples where naloxone-3-glucuronide was not detected, the summed concentration of buprenorphine metabolites was low (41 and 112 µg/L). Both samples were positive for 6-AM, morphine, and codeine, indicating recent heroin use. Many chromatographic peaks were observed in the XIC for naloxone-3-glucuronide between 1-3 min in these samples. These peaks elute near naloxone-3-glucuronide and may interfere with detection, particularly if naloxone-3-glucuronide is present at low concentration in the urine sample. To ascertain whether these interfering compounds were a result of heroin metabolism, urines from individuals taking codeine (identified by a morphine-to-codeine ratio <1, Section 6.6.2.2), heroin (identified by the presence of 6-AM, and a morphine-to-codeine ratio >1), and from an individual known to have consumed poppy seeds were analysed. No interfering peaks were observed in the samples from individuals who had ingested codeine or poppy seeds, but were observed in all the samples from individuals who had recently used heroin (Figure 7.22).

The use of Suboxone as opposed to Subutex may be ascertained through urinary detection of naloxone-3-glucuronide. However, detection of naloxone-3-glucuronide may be difficult in individuals who have recently used heroin due interfering peaks in the XIC.

7.3.8 Identification of Suboxone Adulteration in Urine Samples

Analysis of urine samples from individuals taking Suboxone showed that naloxone was not detected, but most samples contained naloxone-3-glucuronide (Section 7.3.7). Conversely in samples adulterated through addition of Suboxone, a high concentration of naloxone, in addition to buprenorphine, was observed with no glucuronide metabolites present (Figure 7.23).

Figure 7.22 – Extracted ion chromatogram for naloxone-3-glucuronide (*m/z* 504.1864) in patient urines to ascertain the source of interfering peaks

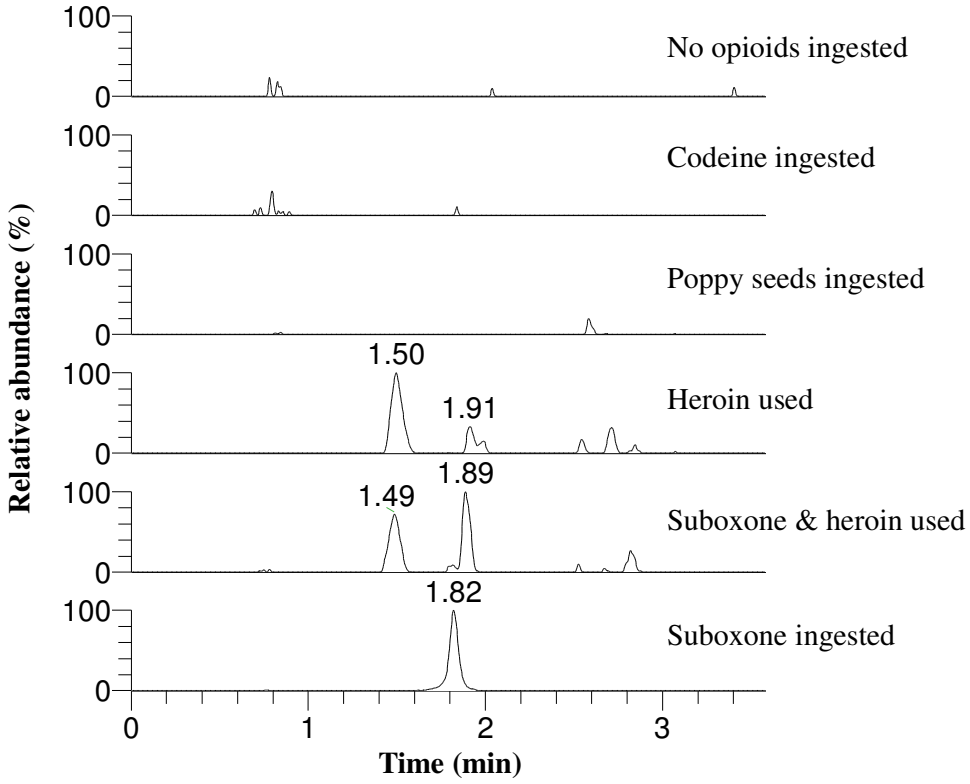
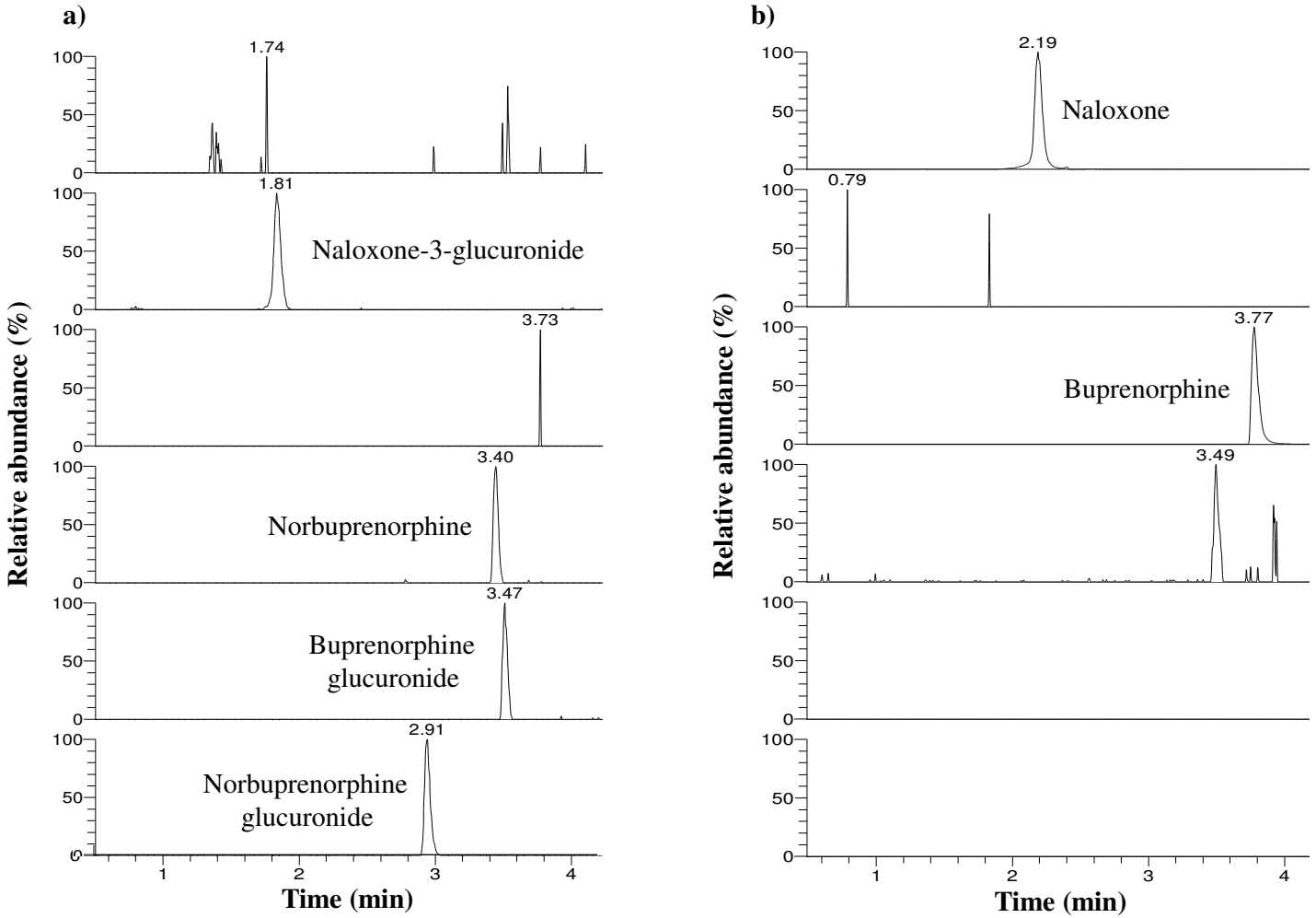


Figure 7.23 – Extracted ion chromatogram of naloxone, buprenorphine, and selected metabolites in patient urine samples to differentiate a) Suboxone ingestion, and b) Suboxone adulteration



7.4 Conclusions

The method developed is simple, robust and rapid. It is capable of detecting and measuring a range of metabolites as well as parent drugs in both plasma and urine. The simultaneous measurement of naloxone and naltrexone in conjunction with this range of metabolites has not been reported before. In addition, the LLoQ for nornaloxone and 6-β-naloxol in plasma has been improved to 0.1 and 0.05 μg/L, respectively, as compared to published methods.

Urinary detection of naloxone and metabolites may be useful to i) detect cases of Suboxone adulteration, and ii) differentiate Suboxone and Subutex ingestion. Detection of naloxone-3-glucuronide is indicative of Suboxone use, provided no naloxone has been administered. A limiting factor is that interference as a result of heroin use may hinder the detection of naloxone-3-glucuronide.

7.4.1 Further Work

Analysis of more urine samples from patients taking Suboxone to ascertain the frequency with which naloxone-3-glucuronide is not detected would help assess whether naloxone-3-glucuronide is a reliable marker for Suboxone use. Ideally the daily Suboxone dose and the time of last dose are required to aid interpretation of results. Whether a correlation exists between either the dose, or the time since last dose, and the concentration of naloxone-3-glucuronide could also be ascertained.

The developed method may be used for analysing the plasma samples collected for naloxone PK studies. A novel buccal naloxone formulation has been developed by King's College London (Alqurshi *et al.*, 2016), and pilot studies to ascertain the PK of naloxone given via this formulation are planned.

8 General Discussion and Conclusions

LC-HRMS methods have been developed for qualitative and quantitative drug detection in biological matrices. The qualitative assay developed for urinary drug detection (Chapter 2) has enabled screening and confirmation of selected drugs to be completed in a single analytical step, which reduces cost and turn-around-time. The method is appropriate for use in a clinical setting, but barbiturates, benzodiazepines, and cannabis assay is still best performed by immunoassay initially. The quantitative assay developed for measurement of naloxone, naltrexone, and selected metabolites (Chapter 7) has good sensitivity, and is suitable for application in PK studies. The method was also used to analyse urine samples, and ascertained that urinary naloxone-3-glucuronide assay may be used to differentiate Suboxone and Subutex use.

8.1 Benefits of Using LC-HRMS for Analysis of Drugs in a Clinical Setting

LC-HRMS analysis has two key advantages in regard to advancing drug detection; the ability to rapidly incorporate new analytes into an existing method, and to retrospectively analyse data. To exploit these benefits fully, sample preparation methods should be kept non-selective to allow as much data as possible to be captured from sample analysis. This approach is well-suited to address the current requirements of a urine drug screening service, where detection of novel drugs may be required in a short time-frame. The other advantage of keeping methods non-selective is that a wider range of analytes may be assayed. By keeping the sample preparation non-selective for the naloxone assay, the more polar analytes (nornaloxone and naloxone-3-glucuronide) could be included in the method.

The established methodology for drugs of abuse testing in most clinical laboratories has not been adapted to account for the change observed in the drug market over recent years. As a result, the use of novel drugs may go undetected. Improving detection of novel compounds is of increasing importance to the scientific community to enable accurate diagnosis and treatment of individuals who abuse these substances. Immunoassays, which are widely used by clinical laboratories for urinary drug detection, may be inappropriate for the detection of NPS. Poor cross-reactivity of mephedrone and its metabolites with the CEDIA amfetamine-group assay has been shown in this study, and analysis of patient samples containing mephedrone in the absence of other amfetamine drugs gives negative results (Chapter 5). The development of immunoassays is a slow process, meaning by the time a kit is commercially available it is likely that targeted drug is no longer in circulation rendering the assay obsolete. On the other hand, LC-HRMS has the advantage of being a versatile and adaptable

methodology which enables the rapid incorporation of NPS into existing methods, as demonstrated through the inclusion of methylphenidate, ethylphenidate, and ritalinic acid, into the drug screening method (Chapter 4).

The ability to retrospectively interrogate data may be used to improve knowledge of a compound's metabolism, particularly for NPS where little is known regarding *in vivo* metabolism. From retrospectively analysing urine samples containing mephedrone, normephedrone, 4-methylephedrine, and 4-methylpseudoephedrine were confirmed as urinary metabolites of mephedrone. In addition, five of the reported urinary metabolites tentatively identified by Pozo *et al.* (2015) were qualitatively identified in most of the urine samples from mephedrone users that were analysed. Two novel urinary metabolites of mephedrone, 4-methylpseudoephedrine and 4-methylpseudo-norephedrine, were also tentatively identified (Chapter 5).

8.2 Limitations of LC-HRMS

Many of the benefits of LC-HRMS can only be exploited if sample preparation is kept as non-selective as possible. However, this may compromise the ability to accurately quantify an analyte as matrix effects are often significant when minimal sample preparation is used. Choosing appropriate internal standards may overcome this problem, as shown through inclusion of naloxone-3-glucuronide-D_3 in the quantitative assay developed. The other impact of minimal sample preparation is that assay sensitivity may be compromised due to ion suppression from matrix components. To an extent, HRMS is able to compensate for this loss by allowing an analytes signal to be extracted from near-isobaric background signals, but in some cases targeted analysis may be necessitated to achieve the required sensitivity.

Whilst there is the capability to retrospectively analyse data, the process is not always straight-forward. The current instrument software is not able to reliably process data, particularly in regard to performing unknown screens, which makes it a time-consuming process. Currently, screening for unknown compounds using LC-HRMS remains a largely manual process and requires skilled analysts to interpret data. Unlike GC-MS, no searchable universal compound library exists for LC-HRMS. The work presented in this thesis is in agreement with Maurer (2013), that the development of an instrument-specific in-house compound library based upon analysis of reference standards is required to enable definitive compound identification. In most cases, results that are generated from retrospective analysis may only be used for qualitative information.

Accurate quantitation is reliant on an appropriate internal standard being included at the time of original analysis, and the analyte concentration being within its linear range. Re-analysis of samples may be undertaken, but the sample may have been discarded or the analyte may have degraded. The long-term physical storage of data may also be problematic due to the vast amount of data generated for each sample.

Whilst the use of HRMS is highly selective, interferences still arise. Isobaric interferences cannot be overcome using mass spectrometry alone, which can make identification of unknown substances difficult. As a result, reference standards are still required for definitive compound identification. Even in cases where a reference material is available for the compound of interest, co-elution of an isobaric compound cannot be excluded. This is particularly relevant for positional isomers, where specific MS^2 product ions often aren't available to differentiate analytes. In this study, measurement of mephedrone in urine samples was undertaken but it could not be excluded that 2-MMC or 3-MMC were actually the compound detected. Non-isobaric interference was also observed from isotopes of analytes, as seen by the M+1 isotope of 4-methylephedrine/4-methylspeudoephedrine and the M+2 isotope of 6-β-naltrexol influencing the apparent peak area of the internal standard. Using selective mass spectrometry (e.g. SRM) may overcome these interferences, provided specific product ions can be identified. However, the capacity for capturing all data from a sample is then lost, and as a result the ability to effectively analyse data retrospectively.

Many drugs, in particular NPS, contain chiral centres. The pharmacological potency of NPS enantiomers may differ as it is already known that other *R*- and *S*- isomers of other drugs possess different efficacy and toxicity profiles (e.g. methcathinone, mephedrone, amfetamine). The development of analytical methods for the chiral separation of NPS may be important in furthering knowledge of these compounds. A limitation of MS is that it is an achiral technique meaning that chiral compounds cannot be differentiated, i.e. the mass spectra of an enantiomeric pair are identical. When coupled with LC, analytical separation of the enantiomers is possible using a chiral stationary phase. However, development of chiral LC methods is often a difficult process.

8.3 Future Opportunities for Drug Screening

Adulteration of samples remains a problem with urine drug screening, with approximately 1 in 50 samples received by our laboratory identified as adulterated through addition of either buprenorphine or methadone. Advances in analytical methods enable identification of these cases through separate detection of parent drug and metabolites, but does not address the main problem. Collection of oral fluid, as opposed to urine, may minimise sample adulteration as oral fluid is a non-private sample. However, other issues such as sample contamination, by sublingual administration of medications (e.g. buprenorphine), and analytical challenges, e.g. low analyte concentration, analyte adsorption to collection devices, need to be overcome.

The use of LC-HRMS for drug screening could warrant a change in reporting results. Results from the audit showed that basing result interpretation on the LoD as opposed to the cutoff concentrations would affect a large number of results. Reporting results based on analyte detection limits enables the window of drug detection to be extended which may be clinically relevant for cases where single or low-dose administration of a drug is suspected (e.g. DFSA, drug administration to children). In addition, routinely reporting to the limit of detection may be beneficial for selected analytes, most notably 6-AM. The audit showed that through reporting the presence of 6-AM based on the LoD, an additional 93 cases of recent diamorphine or heroin use could have been identified which may have an impact on the clinical management of an individual.

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Appendices

Appendix A

Mephedrone Assay: x-y and Bland-Altman plots comparing the concentrations calculated when using either mephedrone-D_3 (sample re-analysis), or codeine-D_6 (retrospective analysis) as internal standards (see Section 5.3.2)

Included herein:

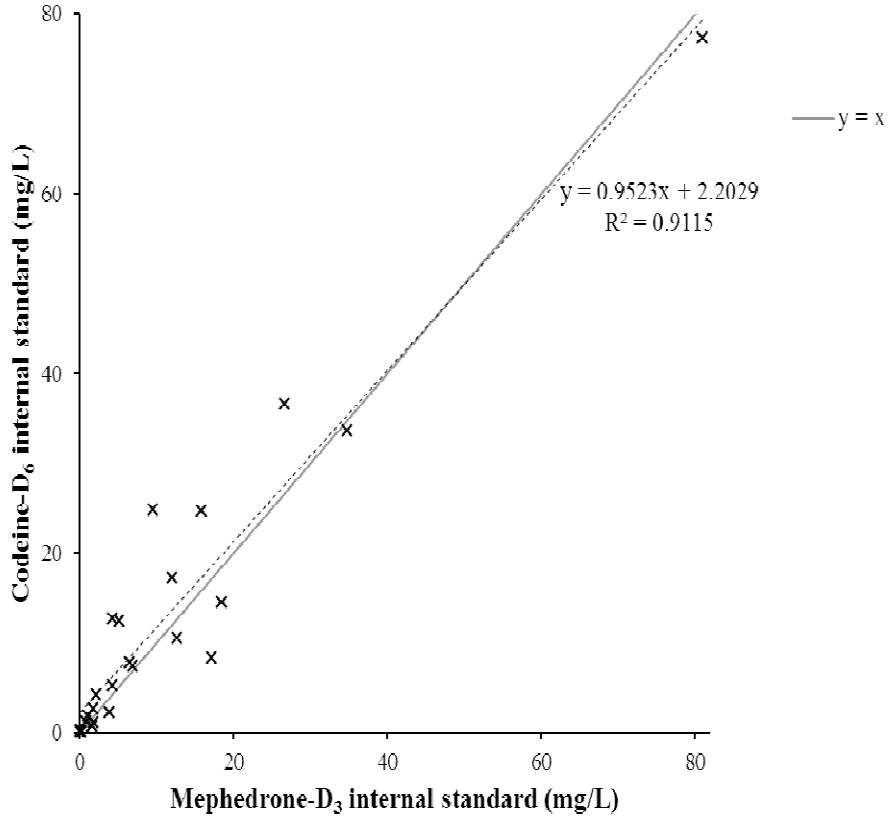
Figure A-1: Mephedrone

Figure A-2: Normephedrone

Figure A-3: 4-Methylephedrine

Figure A-4: 4-Methylpseudoephedrine

Figure A-1: Mephedrone



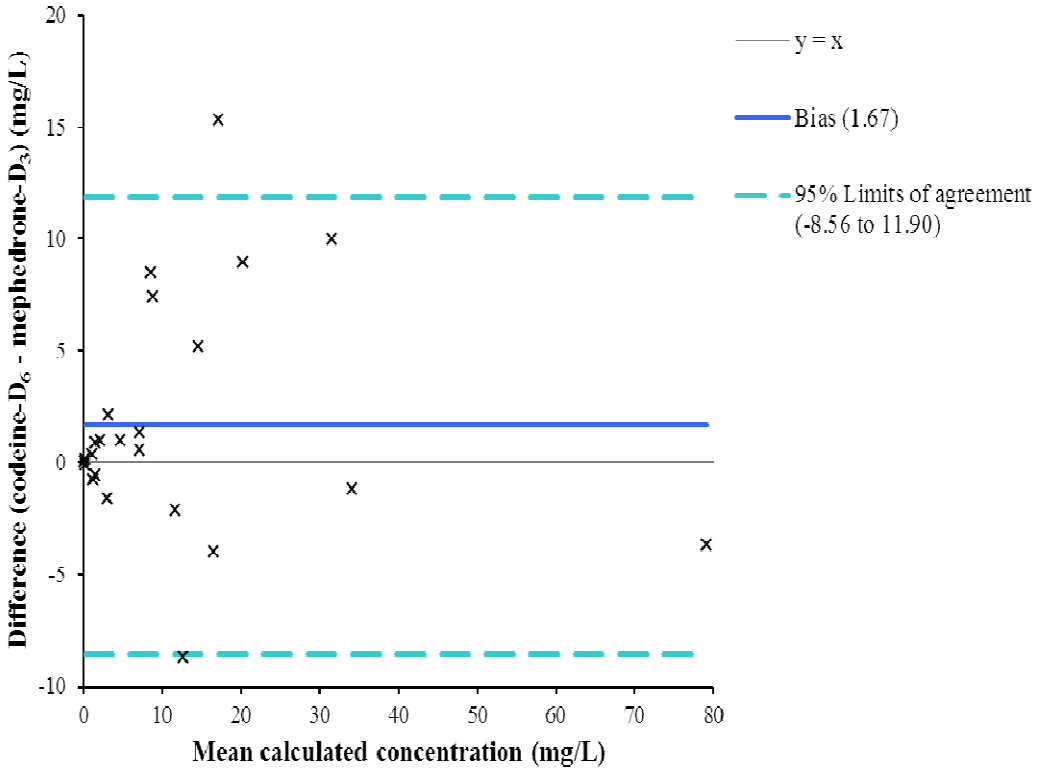
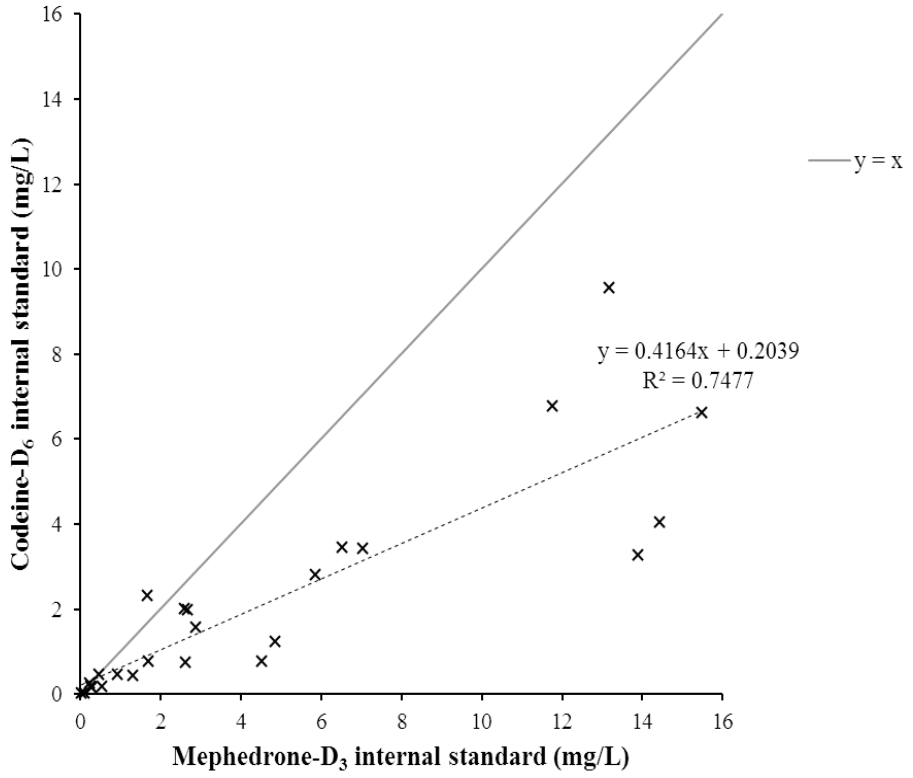


Figure A-2: Normephedrone



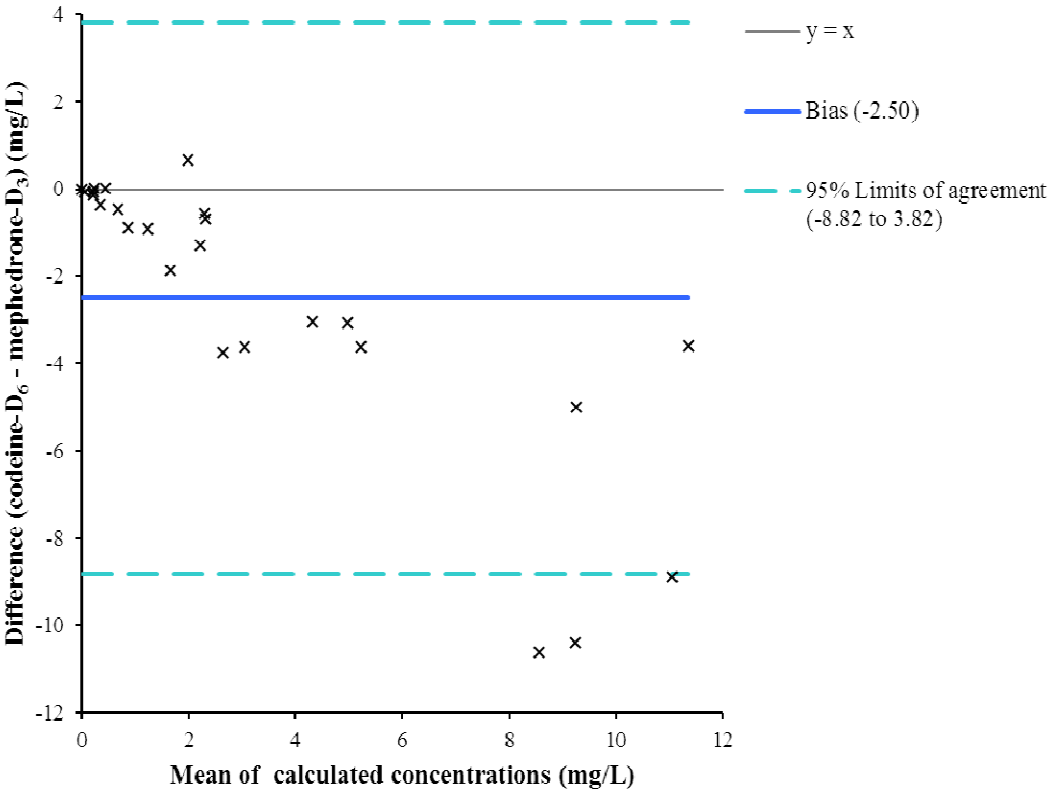
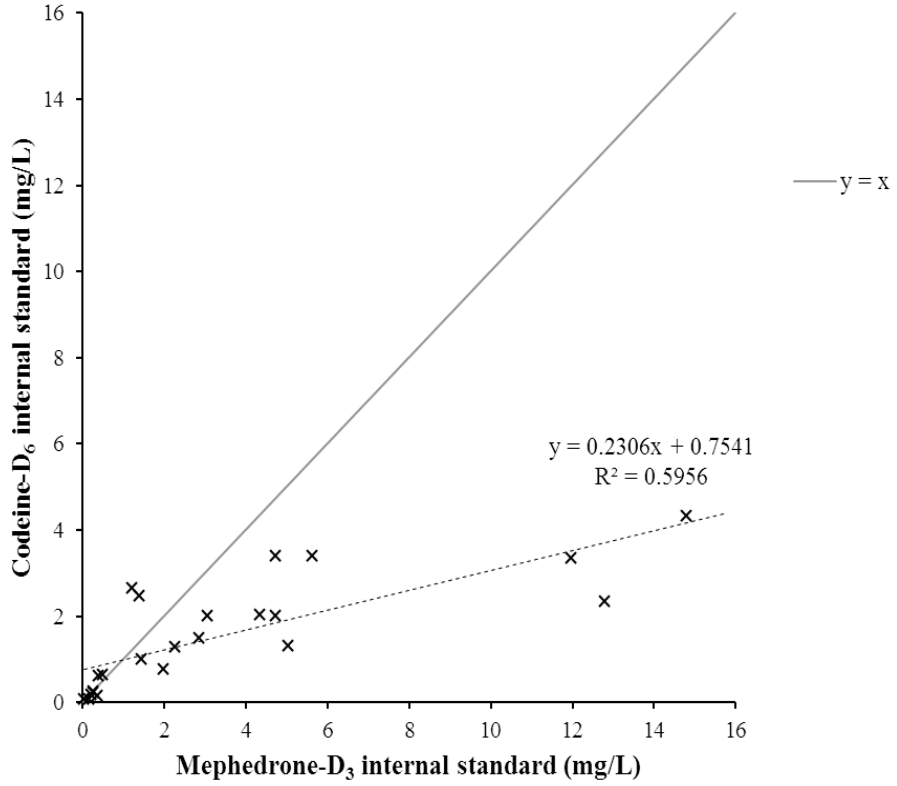


Figure A-3: 4-Methylephedrine



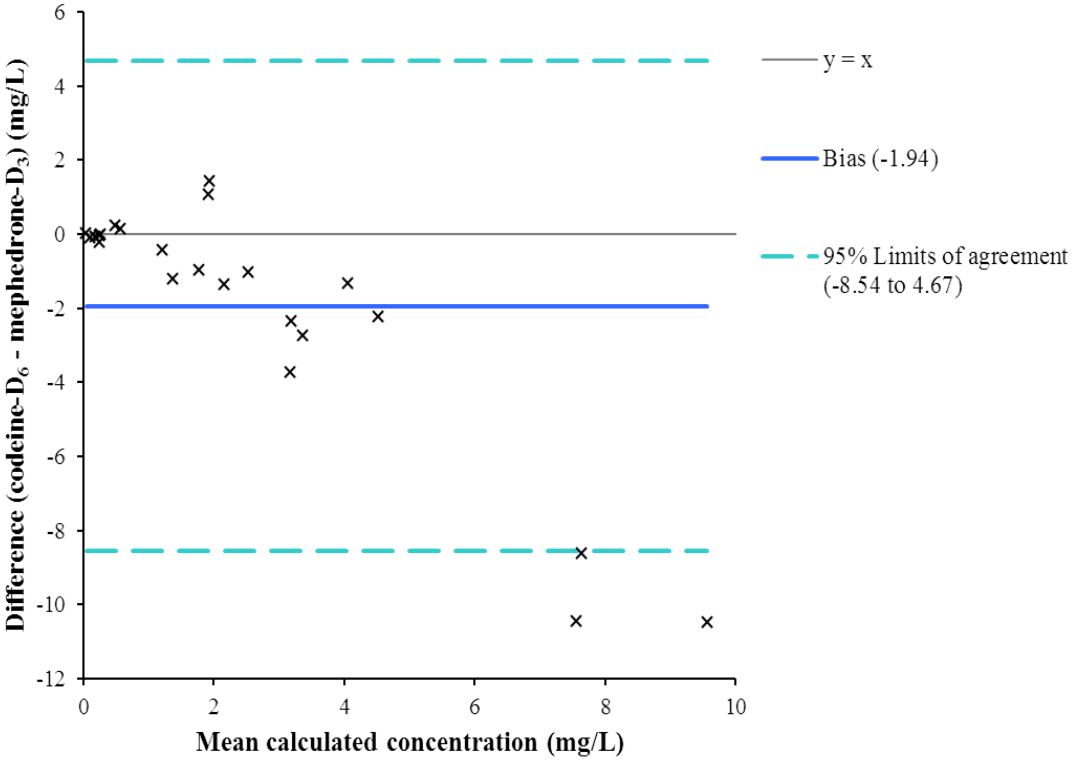
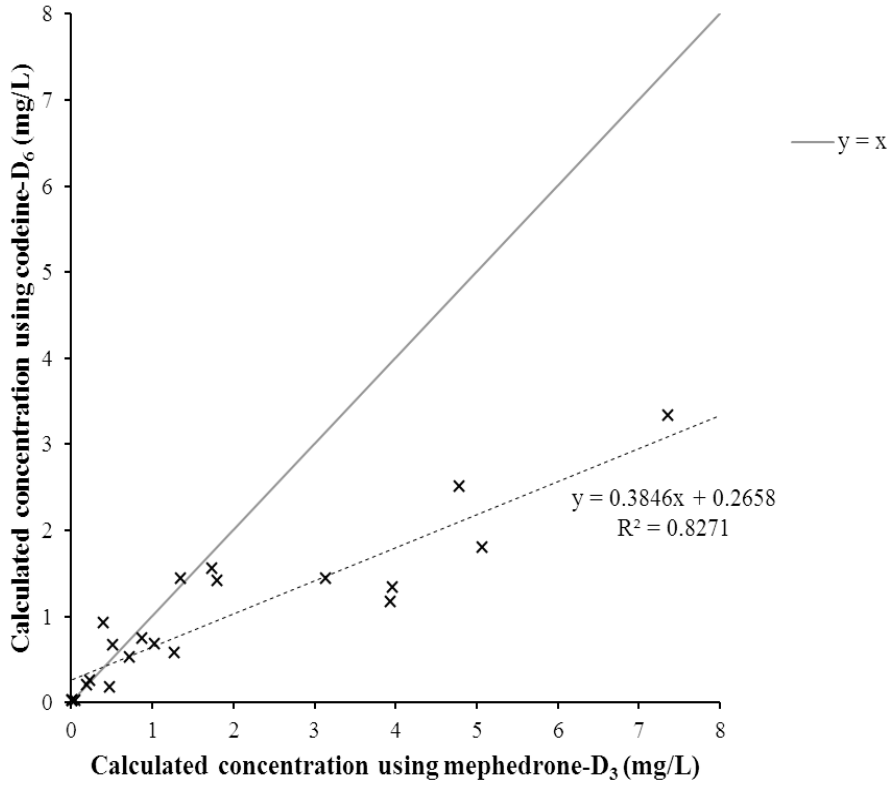
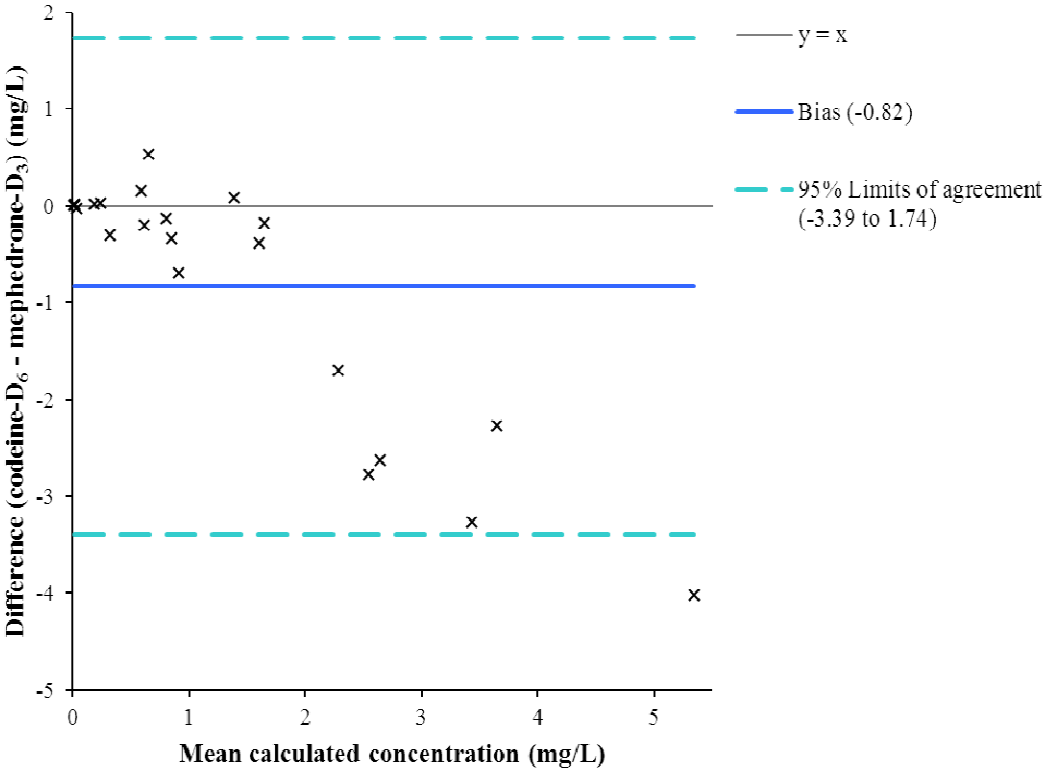


Figure A-4: 4-Methylpseudoephedrine





Appendix B

Mephedrone Assay: x-y and Bland-Altman plots to compare the analyte concentrations calculated when analysing undiluted and diluted urines (see Section 5.3.3)

Included herein:

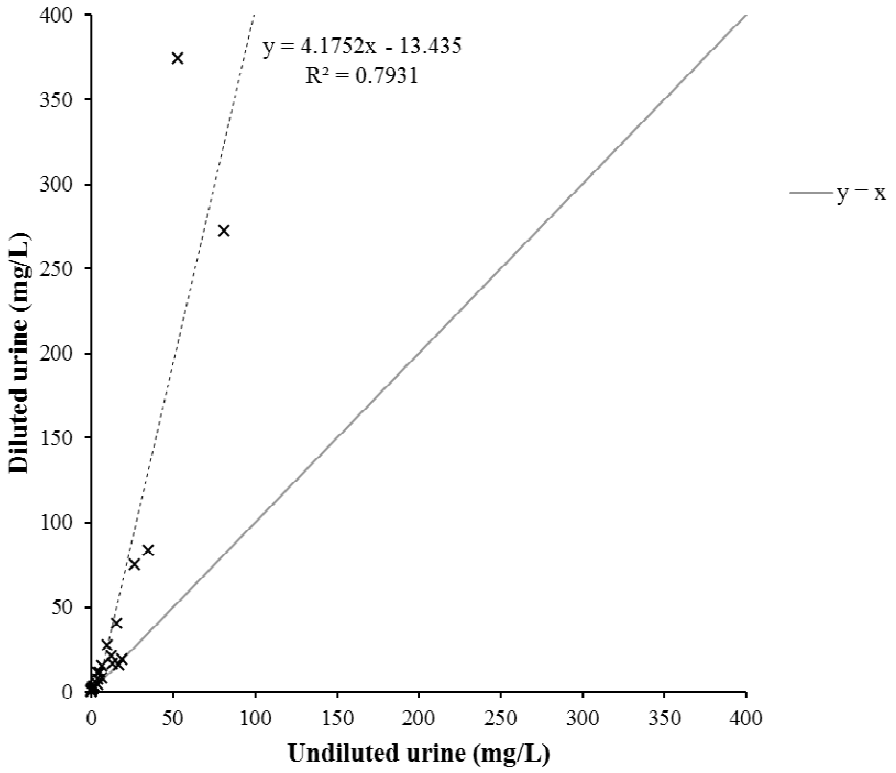
Figure B-1: Mephedrone

Figure B-2: Normephedrone

Figure B-3: 4-Methylephedrine

Figure B-4: 4-Methylpseudoephedrine

Figure B-1: Mephedrone



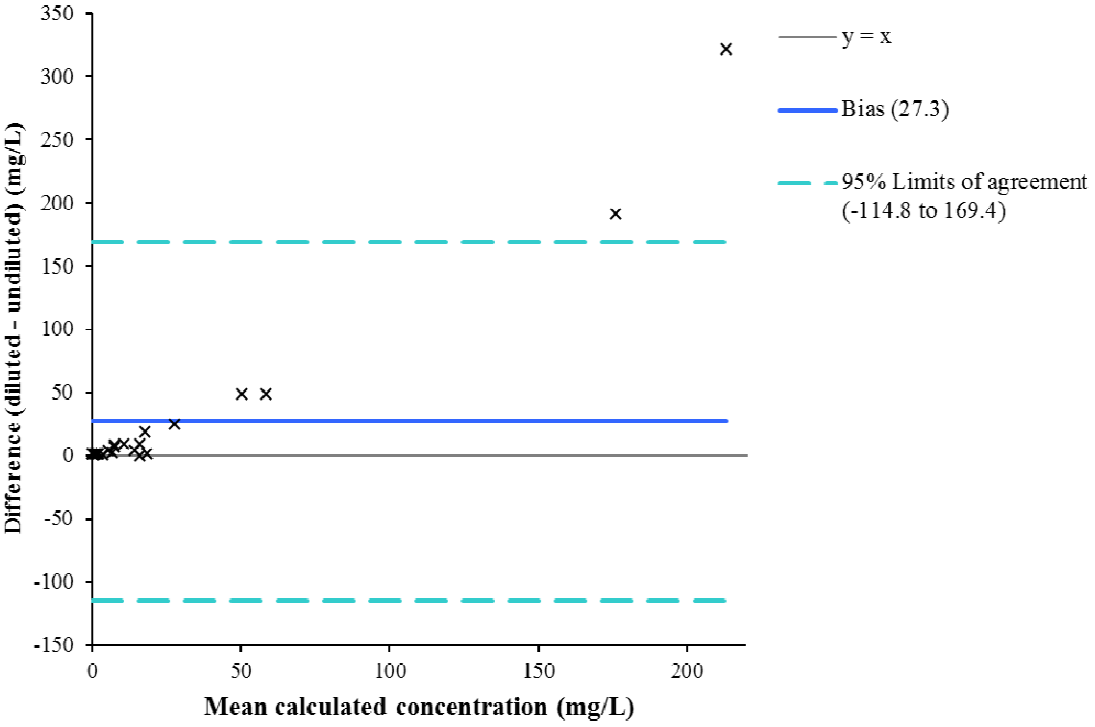


Figure B-2: Normephedrone

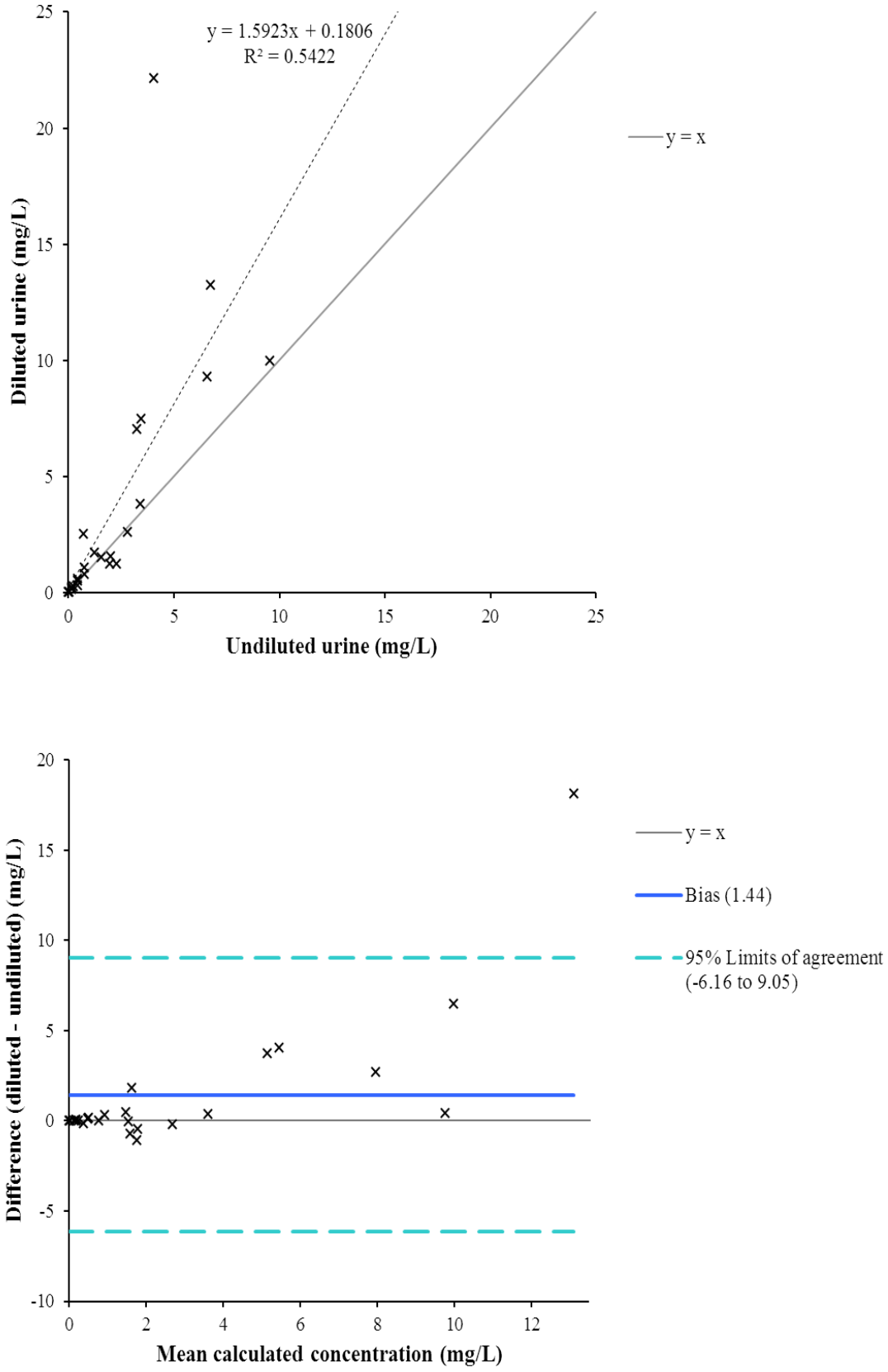
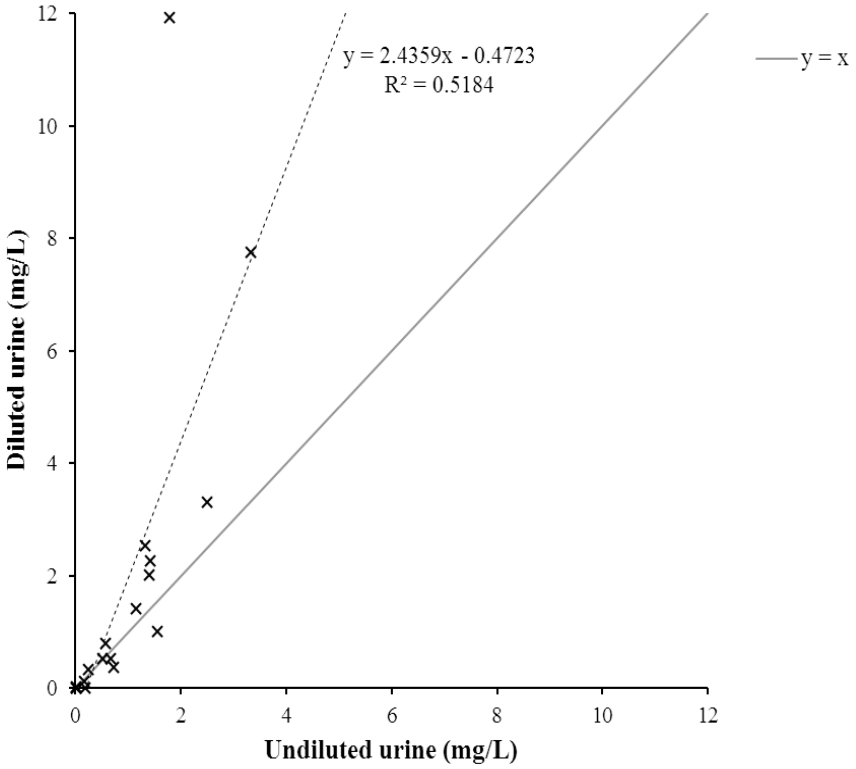


Figure B-3: 4-Methylephedrine



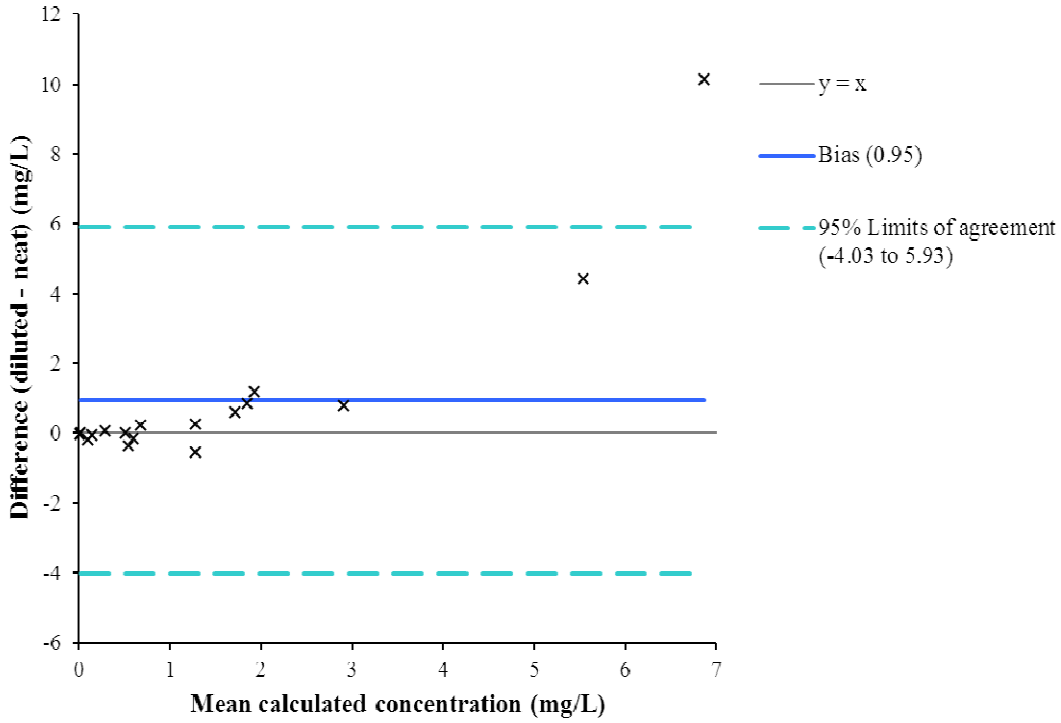
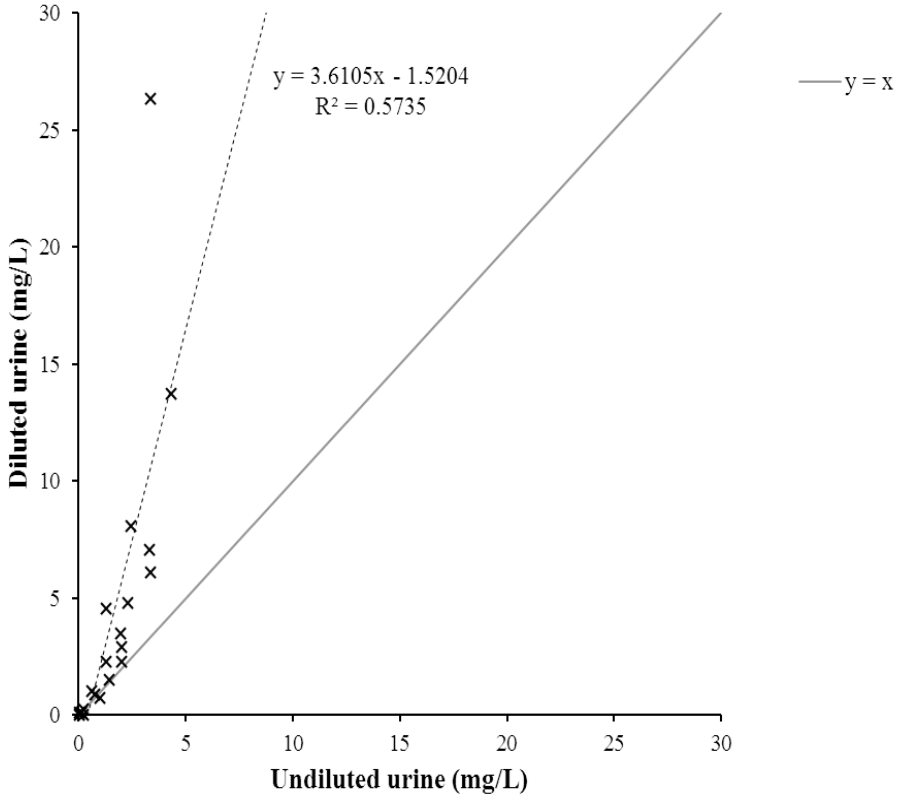
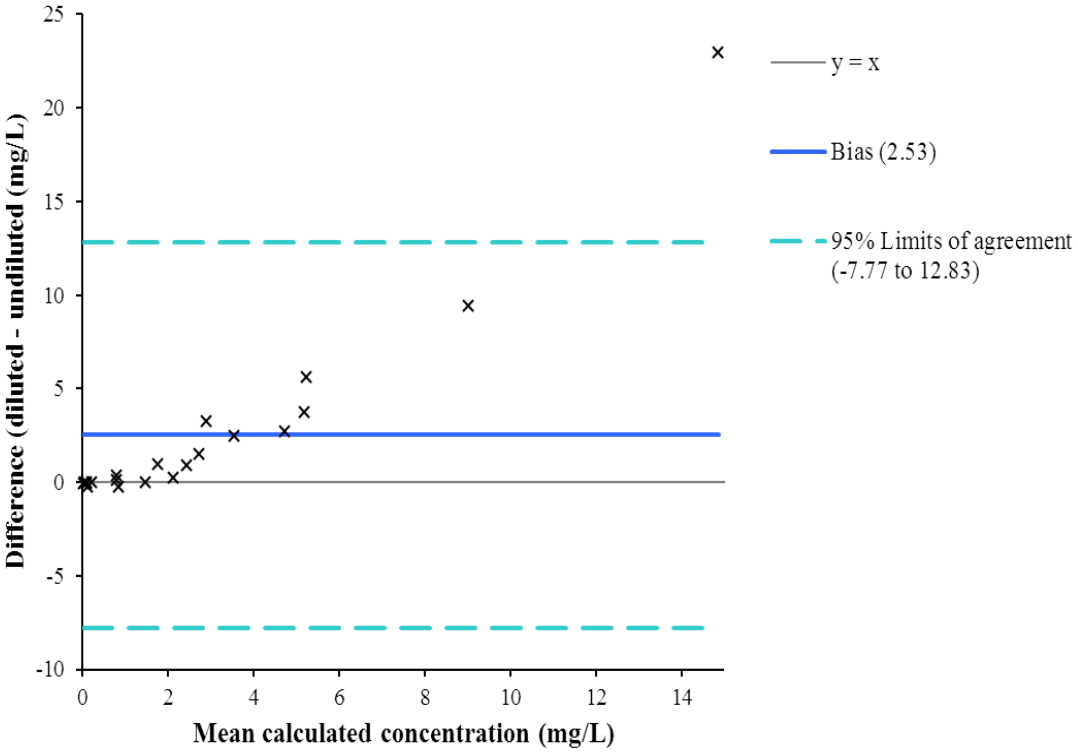


Figure B-4: 4-Methylpseudoephedrine





Appendix C

Publications

Included herein is a complete copy of a relevant publication produced prior-to this project:

- Belsey S.L., Couchman L., and Flanagan R.J. (2014). Buprenorphine detection in urine using liquid chromatography-high-resolution mass spectrometry: comparison with cloned enzyme donor immunoassay (ThermoFisher) and homogeneous enzyme immunoassay (Immunalysis). *J. Anal. Toxicol.* **38**, 438–443.

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